

## Article

# Origin of Wild Polyploid *Avena* Species Inferred from Polymorphism of the ITS1 rDNA in Their Genomes

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**Abstract:** In this article, we analyzed the origin of wild polyploid oats (*Avena* L., Poaceae) using the region 18S rDNA (partially)–ITS1–5.8S rDNA obtained via NGS. There are six tetraploid (2n = 28) and four hexaploid (2n = 42) wild species differing by specific genome combinations: *A. barbata*, *A. vaviloviana* (AB), *A. agadiriana* (AB or BB), *A. magna*, *A. murphyi*, *A. insularis* (AC or CD), *A. ludoviciana*, *A. sterilis*, *A. fatua*, and *A. occidentalis* (ACD). We compared the pool of marker sequences of polyploid oats with those of their putative diploid ancestors: *A. atlantica* (As-genome), *A. hirtula* (As), *A. canariensis* (Ac), *A. ventricosa* (Cv), and *A. clauda* (paleopolyploid with Cp and A-related rDNA). We found 15 major ribotypes (more than 1000 reads per rDNA pool) in polyploid oats. Comparing them, we found that the AB-tetraploid oats possibly inherited their A-genome ribotypes from *A. atlantica* (As1-ribotype), whereas their B-genome ribotype is specific and can be a derivative of the A-genome family. Our data do not support the hypothesis of the CD-genome set in *A. magna*, *A. murphyi*, and *A. insularis*: they have an AC-genome ribotype constitution instead. The C-genome-related sequences could have been obtained from *A. ventricosa*. Hexaploids show a different ribotype pattern than tetraploids; the main ribotypes of *A. fatua*, *A. ludoviciana*, and *A. sterilis* probably belong to the D-group and are also shared with one of the major ribotypes of *A. clauda*.



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## 1. Introduction

The genus *Avena* L. (Poaceae) is one of the most economically important cereals. It comprises about 30 species that form a polyploid row (from diploids, 2n = 14, to hexaploids, 2n = 42). All wild oats historically belong to the ancient Mediterranean region and, as many researchers suppose, originated from multiple hybridizations. Recent cytogenetic and molecular phylogenetic studies have described three genome types that characterize the oat species: A, B, C, and D. The *Avena* ancestor probably have had a diploid chromosome set with “symmetrical” chromosomes, similar in this respect to the karyotypes of diploid oats with the A genome and that of *Arrhenatherum* P. Beauv. [1], as well as to the chromosome sets (CmCm) of *A. macrostachya* Balansa & Durieu. Further, there was a divergence of oat phylogenetic lines with the genomes A and C, accompanied by the accumulation of differences in dispersed repeats (apparently determining the results of GISH hybridization) and the accumulation of transitions and transversions specific to each branch. According to cytogenetic data [2] the A-genome oats have a symmetrical karyotype, whereas in *Avena* species, with the C-genome, chromosome rearrangements occurred, resulting in the appearance of multiple small heterochromatin blocks and desymmetrization of chromosomes. From a cytological point of view, the A-genome oats can have a more primitive karyotype [2] than the C-genome species, which tend to have more primitive morphological

features [3]. This pattern indicates evolutionary heterogeneity: taxa evolve rather slowly, retaining archaic morphological features, but cannot be called truly primitive [4].

Considering the evolution of tetraploid oat species, we can trace two main independent lines: species with AB genomes and species with AC genomes. The genomic structure of the AB-genome oats probably derives from the As-genome variant [2,5–7] though some other researchers consider the Ac-genome oat to be the progenitor of the AB-genome tetraploids [3,8]. The B-genome in oats is known only in tetraploids and does not occur in diploids. This genome variant is thought to be a derivative of the A-genome [9–11]. Probably, the AB-genome species originated from the As-genome species via autopolyploidization [6,12], and thus can be considered the species with the AA' genome. The AC-genome oats show a close relationship with the hexaploid ones [13]. In accordance with cytogenetic data, some researchers considered *A. insularis* Ladiz. as the tetraploid progenitor of hexaploid *Avena* species [3,13–15], while others regarded *A. maroccana* Gand. (= *A. magna* Murphy & Terrell) and *A. murphyi* Ladiz. as possible ancestors of hexaploids [7]. The C-genome in the AC-genome tetraploids has probably been inherited from *A. ventricosa* Balansa [11] or from *A. clauda* Durieu [16]. In addition to some challenges about relationships between tetraploids and hexaploids, there are also ambiguities concerning their very genomic constitution. Some previous studies determined the tetraploids with the C-genome as AC [17]. However, later works based on Acc-nuclear and chloroplast gene sequences suggested that these tetraploids are in fact the bearers of the CD-genome [18,19]. The D-genome was thought to be related to the A-variant [20,21].

Evolution of hexaploid *Avena* species are the most complicated; the A-genome of hexaploid oats is different from that of diploid species [22]. Cytogenetic research also showed that the putative A-genome donor to hexaploid oats could have been tetraploid *A. magna* [23]. Recent phylogenetic studies estimate some Al/Ad-genome species as the A-genome ancestor of hexaploids, whereas the C and D-genomes were obtained by them from some CD-tetraploids [19]. The D-genome diploid progenitor may be extinct according to the genomic studies [19]. In addition, *A. sterilis* L. is thought to be the first in the hexaploid oat line and the progenitor of all their diversity [3,11,24]. *Avena occidentalis* Durieu, a relic of the ancient African flora, is probably a relative of *A. vaviloviana* (Malzew) Mordv. (the AB-genome tetraploid) and thus can represent the second ancestral line of hexaploid oats [11,25].

As we see, there are some questions about the origin of hexaploid oats and the evolutionary connections between diploids and tetraploids. Besides, we earlier described possible introgression cases within the diploid *Avena* [26,27]. These introgression events occurred not only within the A- and C-genome species but also between them [26,27]. *Avena clauda* (previously determined as the Cp-genome species) has three major ribotypes related to the A-genome, especially one that is shared with *A. longiglumis* Durieu (the ribotype of the minor fraction) [27]. Thus, drastic differences between the A- and C-genome compositions did not prevent the introgression between the genome bearers. Also, we cannot exclude the possible evolution of the A-genome from the C-genome ancestor close to modern *A. clauda*.

Locus-specified next-generation sequencing can reveal hidden hybridization when morphological features are constant and do not identify the hybrid origin [28]. For our aims, we took marker sequences including 18S–ITS1–5.8S rDNA. This region can be a suitable marker in cases of multiple hybridization between related species and is sometimes more effective than ITS2 because of its conservative structure and higher substitution rate [29–31]. Our goal in this research is to find possible ancestral species of polyploid oats among diploid ones and trace evolutionary pathways that could lead to the origin of hexaploids.

## 2. Results

The aligned sequences were sorted into ribotypes with a certain amount of reads. Major ribotypes were the ribotypes that were presented in the rDNA pool more than 1000 times. Minor ribotypes with a threshold of 10 reads per whole pool were usually variants of the major ribotypes in the case of the A-genome. The C-genome-related sequences were also in

the minor fraction (from 10 to 40 reads). Table 1 demonstrates the studied species and their ribotype constitution.

Table 2 summarizes the major ribotypes of polyploid oats and their possible diploid ancestors: *A. atlantica* B.R. Baum and Fedak, *A. canariensis* B.R. Baum, Rajhathy, and D.R. Sampson, *A. hirtula* Lag. (A-genome oats), *A. ventricosa*, and *A. clauda* (C-genome species, paleopolyploid). We took as the consensus sequence that of *A. atlantica* (As-genome), a possible ancestor of the A-genome component in the polyploid species.

The majority of the differences between ribotypes are located within the ITS1 region; the differences are represented by SNPs (single nucleotide polymorphisms) and deletions.

We analyzed three datasets obtained via NGS. The first dataset was aimed at studying the phylogeny of tetraploid oat species. It contains 660 sequences of tetraploids and their possible diploid, A-genome donors, *A. atlantica* and *A. canariensis* (Supplementary Table S1). The second dataset comprises sequences of hexaploid ACD-oats, their putative tetraploid progenitors of the AC-genome, and diploid species *A. atlantica*, *A. canariensis*, and *A. ventricosa*. It consists of 792 sequences of 18S rDNA–ITS1–5.8S rDNA (Supplementary Table S2). The third dataset contains the marker sequences of hexaploid oats, related tetraploids, and sequences of the diploid *A. hirtula* and paleopolyploid *A. clauda* (917 accessions, Supplementary Table S3).

Totally, we found 23 major ribotypes in the studied *Avena* species. Networks built by the statistical parsimony algorithm are presented in Figure 1, Figure 3, and Figure 5. *Avena canariensis*, a possible progenitor of the polyploid species [3,8,11,14] has two related major ribotypes that did not participate in the formation of AB and AC-polyploids (Figure 1a,b).

*Avena atlantica*, on the other hand, shares its two major ribotypes, As1 (6353 reads, 39% of the rDNA pool) and As2 (4571 reads, 28%), with tetra- and hexaploid oats. The main ribotype of the AB-genome species *A. barbata* Pott ex Link is As1 (4894 reads, 33% of the rDNA pool). The same main ribotype was found in *A. vaviloviana* (10,884 reads, 47%). The second major ribotype of *A. barbata* (AB-genome) is species-specific (B4, 1014 reads, 6%). The AB-genome oat *A. agadiriana* B.R. Baum & Fedak has a species-specific main ribotype B1 (7136 reads, 28%), and its ribotype As2 is only the fourth of most represented (1018 reads, 4%). The second of the major ribotypes in *A. agadiriana* is B2 (4919 reads, 41%); it is shared with the minor ribotype fractions of *A. atlantica* (15 reads) and *A. magna* (18 reads). The third major ribotype of *A. agadiriana* is species-specific (1275 reads, 5%). Two well-represented ribotypes of the AB-genome species *A. vaviloviana*, B5 (3255 reads, 14%) and B6 (1162 reads, 5%), are shared with *A. barbata* (as minor ribotypes of it).

All the AC-genome oats have at least one A-genome ribotype inherited from *A. atlantica*—the second variant, As2 (Figure 1a,b). This is the main ribotype in the AC-genome oats *A. insularis* and *A. murphyi* (8297 reads, 39% and 6900 reads, 44%, respectively). It is also the third of major ribotypes in *A. magna* (3847, 15%). The latter species has a species-specific A-related main ribotype (Am1, 6067 reads, 24%). In addition, the second major ribotype of *A. insularis* is species-specific (Ai, 3770 reads, 18%). *Avena murphyi* has the second major ribotype, Amp (4443 reads, 28%), that belongs to the A-related group (Figure 1, Table 2). It is common in a minor fraction of ribotypes of *A. magna* (467 reads), *A. insularis* (196 reads), and *A. agadiriana* (33 reads). In these species, the C-genome sequences remained only in a minor fraction of the rDNA pool (from 78 to 10 reads, Figure 1a,b). The ribotype tree obtained by Bayesian inference (Figure 2) shows the clear distinction of the AC-genome sequences (*A. canariensis*) from other A-genome oats (PP = 0.86, BS = 93), except for two minor ribotypes.

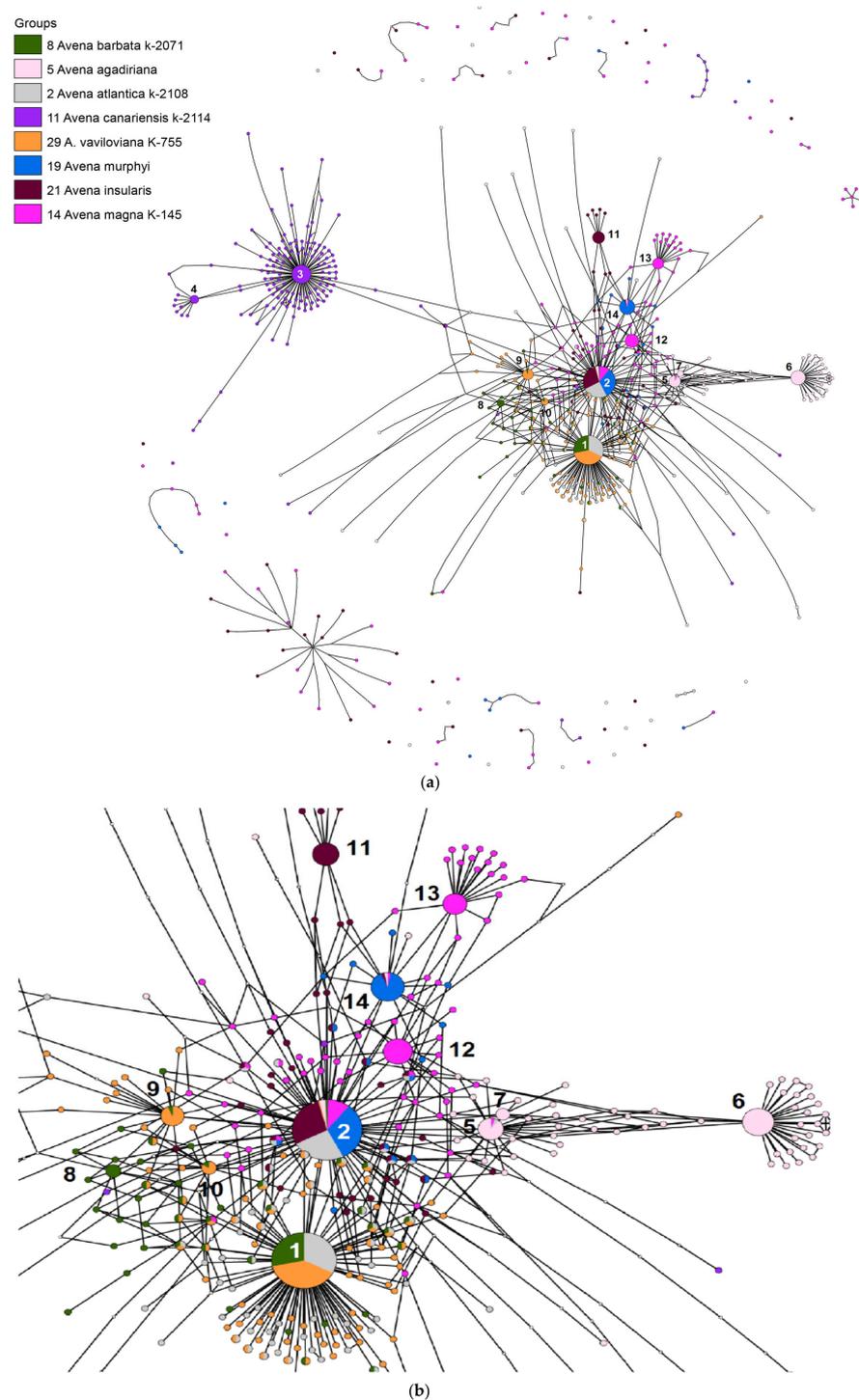
**Table 1.** Summary of the oat species (polyploids and possible parental taxa) used in the present study and their genome types and ribotypes.

Species	Sample ID	Country of Origin	Accession Number	Collected by	Genome	Total Number of Reads	Ribotype Number in Figures 2 and 4	Ribotype Symbol (of the Major Ribotypes)	Number of Reads	% from the Total Number of the Reads
<i>Avena atlantica</i>	K-2108	Morocco	from OM004717 to OM004731; from OQ338511 to OQ338567	A. Diederichsen	As	16,241	1	As1	6353	39
							2	As2	4571	28
<i>Avena canariensis</i>	K-2114	Spain	from OM004567 to OM004604; from OQ338401 to OQ338510	A. Diederichsen	Ac	20,606	3	Ac1	10,050	49
							4	Ac2	1908	9
<i>Avena agadiriana</i>	K-2123	Morocco	from OQ135344 to OQ135431		AB?	25,194	5	B1	7136	28
							6	B2	4919	41
							7	B3	1275	5
							2	As2	1018	4
<i>Avena barbata</i>	K-2071	Lebanon	from OQ135432 to OQ135477		AB	14,896	1	As1	4894	33
							8	B4	1014	6
<i>Avena vaviloviana</i>	K-755	Ethiopia	from OQ135478 to OQ135562		AB	23,292	1	As1	10,884	47
							9	B5	3255	14
							10	B6	1162	5
<i>Avena insularis</i>	K-2067	Italy	from OQ135592 to OQ135663		AC or CD	21,046	2	As2	8297	39
							11	Ai	3770	18
							12	Am1	6067	24
<i>Avena magna</i>	K-145	Morocco	from OQ135664 to OQ135782	E. V. Mazhorov	AC or CD	25,167	13	Am2	4032	16
							2	As2	3847	15
<i>Avena murphyi</i>	K-2088	Spain	from OQ135563 to OQ135591		AC or CD	15,596	2	As2	6900	44
							14	Amp	4443	28
<i>Avena fatua</i>	K-31	Kazakhstan	from OQ144753 to OQ144793		ACD	12,280	15	D	5203	42
							14	Amp	1608	13

Table 1. Cont.

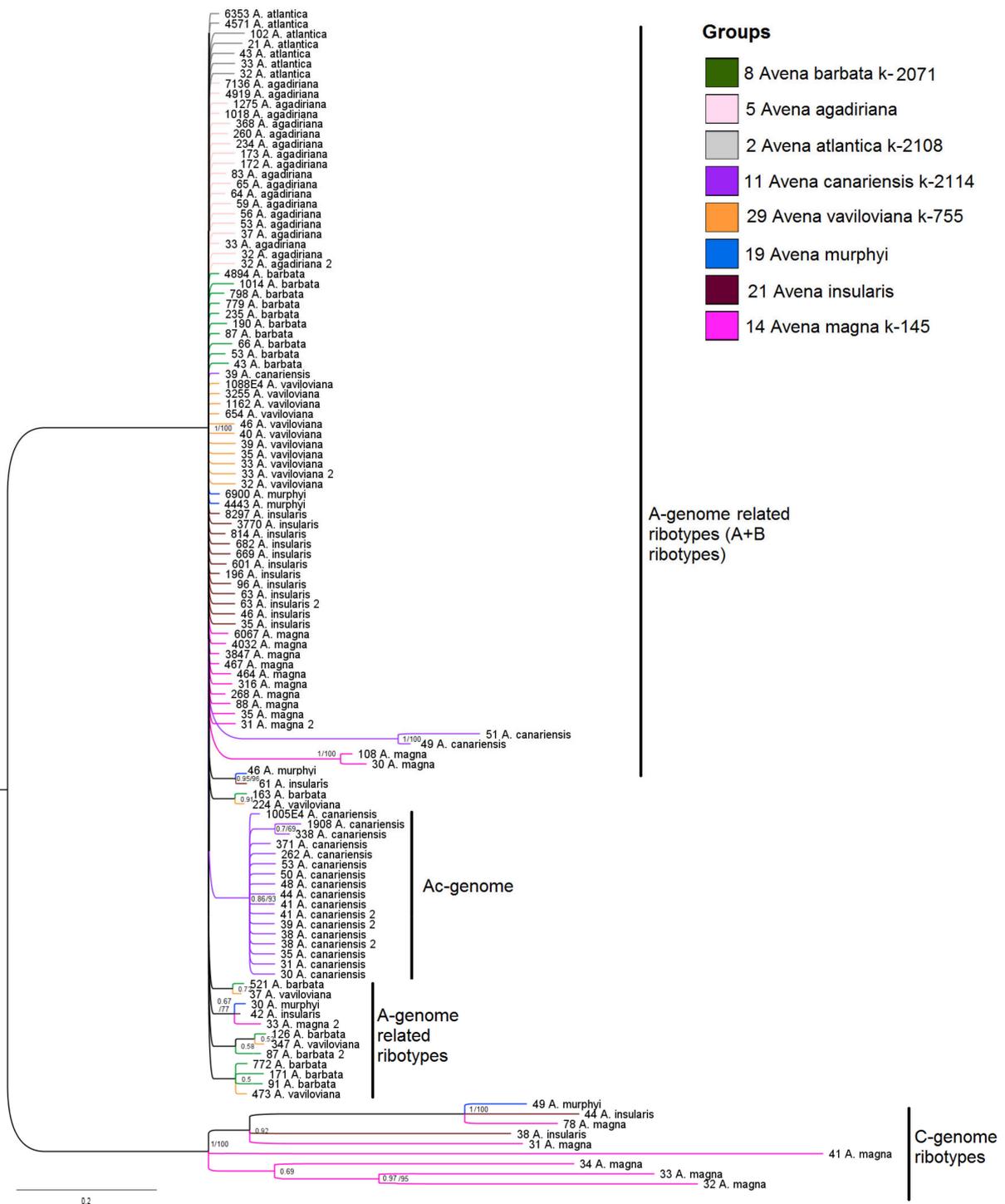
Species	Sample ID	Country of Origin	Accession Number	Collected by	Genome	Total Number of Reads	Ribotype Number in Figures 2 and 4	Ribotype Symbol (of the Major Ribotypes)	Number of Reads	% from the Total Number of the Reads
<i>Avena ludoviciana</i>	K-383	Russian Federation	from OQ144842 to OQ144891		ACD	15,740	15	D	4682	30
							16	Ald/Cc2A	3811	24
							14	Amp	1409	9
<i>Avena occidentalis</i>	K-1967	Spain, Canary is.	from OQ144691 to OQ144752		ACD	21,870	15	D/Cc2C	4668	21
							17	Ast (D'?)	3763	17
							14	Amp	2694	12
<i>Avena sterilis</i>	K-323	Russian Federation	from OQ144794 to OQ144841		ACD	13,147	17	Ast (D'?)	6138	47
							14	Amp	1820	14
<i>Avena ventricosa</i>	K-2056	Algeria	from OK301935 to OK302014; from OQ520145 to OQ520214		Cv	15,506	18	Cv	12,560	81
<i>Avena clauda</i>	K-269	Azerbaijan	from OK273905 to OK274031; from OQ834465 to OQ834560	V. N. Soldatov	Cp	40,168	19	Cc1A	5320	13
							20	Cc1B	2695	7
							16	Ald/Cc2A	3195	8
							21	Cc2B	1777	4
							15	D/Cc2C	1305	3
<i>Avena hirtula</i>	k-1878	Spain	from OM004668 to OM004716; from OQ834671 to OQ834744	M. Leggett	As	31,625	22	As5	12,605	40
							23	Ad2/As3	3587	11





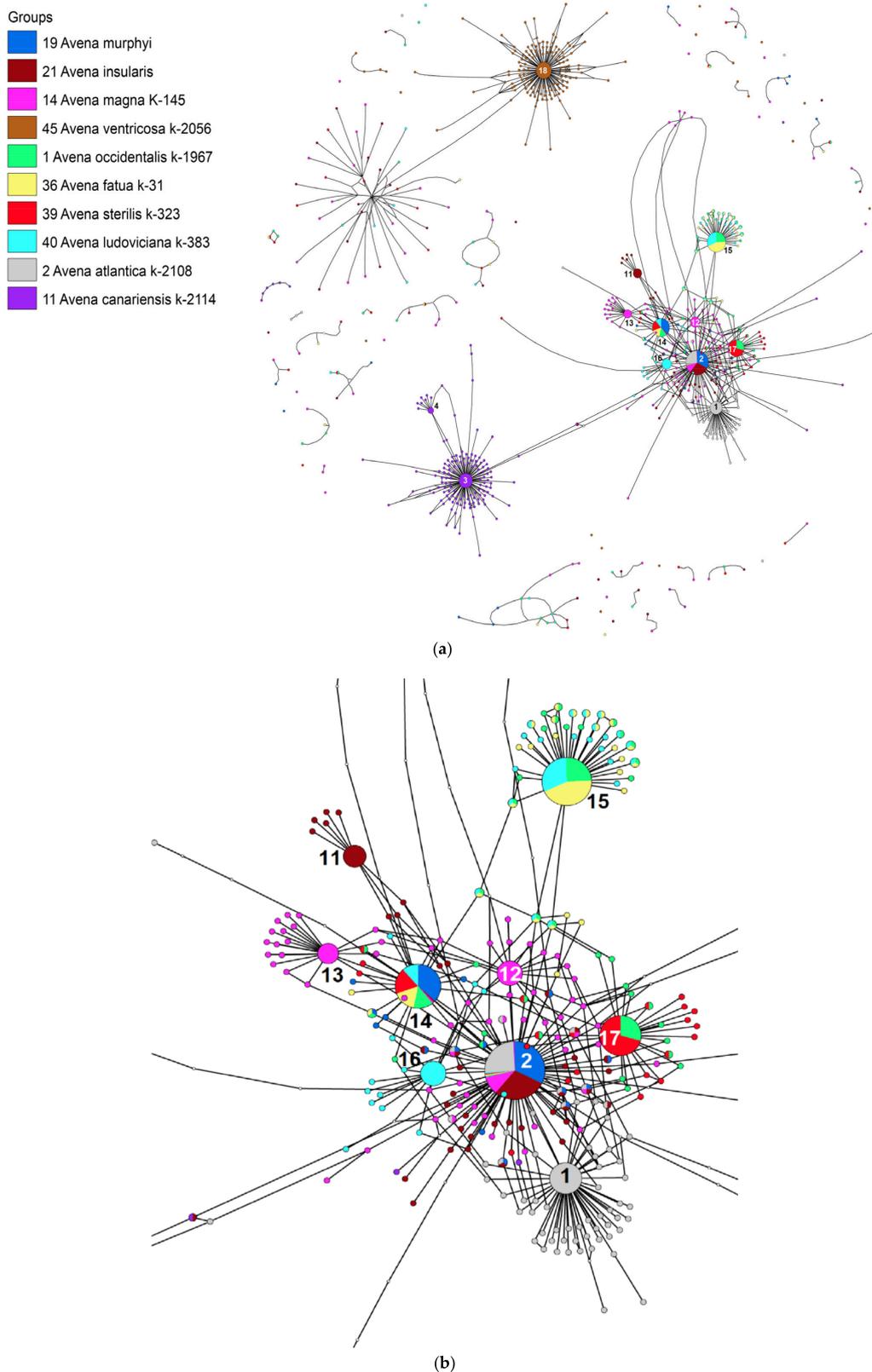
**Figure 1.** (a) Ribotype network of wild tetraploid *Avena* species and their putative parental taxa. The radius of the circles on the ribotype network is proportional to the percent number of reads for each ribotype, as shown in Table 1. Major ribotypes are larger than others (more than 1000 reads per rDNA pool) and marked with numbers. The smallest circles correspond to ITS1 variants that have been read fewer than 1000 times. (b) Ribotype network of wild tetraploid *Avena* species and their putative parental taxa. More detailed picture of the major ribotypes.

The C-genome-derived sequences of the AC-genome, in which the largest number of reads is 78 (PP = 1, BS = 100), form a separate cluster. The B-genome-related ribotypes in the tree do not separate from the A-related ones (Figure 2).



**Figure 2.** Phylogenetic tree of the ribotypes of wild tetraploid *Avena* species and their putative parental species. The first index on the branch is the posterior probability in Bayesian inference, and the second is the bootstrap index obtained by the Maximum Likelihood algorithm. When only one index is shown on the branch, it is the posterior probability. Numbers before the species name indicate the number of reads per rDNA pool.

The second network (Figure 3a,b) depicts the relationships among hexaploids and their possible parental taxa. The main ribotypes of hexaploid oats *A. fatua* L., *A. ludoviciana* Durieu, and *A. occidentalis* (5203 reads, 42%; 4682 reads, 30%; and 4668 reads, 21%, respectively) are common and belong to a single family that does not occur in the tetraploids.



**Figure 3.** (a) Ribotype network of wild hexaploid *Avena* species and their putative parental taxa. The radius of the circles on the ribotype network is proportional to the percentage of reads for each ribotype. Major ribotypes are larger than others (more than 1000 reads per rDNA pool) and marked with numbers. (b) Ribotype network of wild hexaploid *Avena* species and their putative parental taxa. More detailed picture of the major ribotypes.

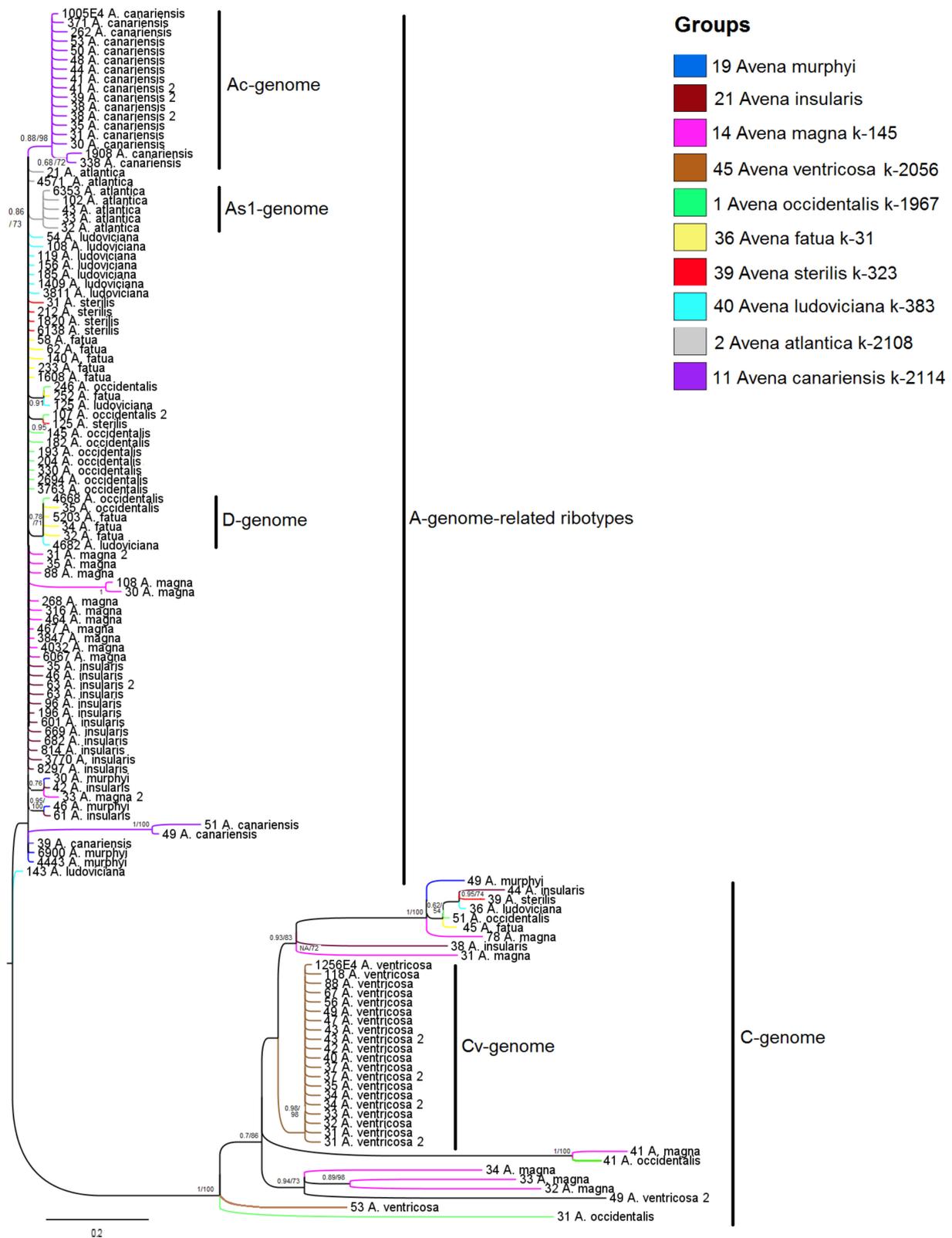
These ribotypes can represent the D-genome. The A-related ribotypes of *A. fatua* (1608 reads, 13%), *A. ludoviciana* (1409 reads, 9%), and *A. occidentalis* (2694 reads, 12%) are homologous with the second major ribotype of *A. murphyi* (Amp). At the same time, *A. sterilis* and *A. occidentalis* have one common ribotype that can be named Ast. It is the main ribotype for *A. sterilis* (6138 reads, 47%) and the second for *A. occidentalis* (3763 reads, 17%). One of major ribotypes of *A. ludoviciana* (Ald, 3811 reads, 24%) is species-specific and closely related to the main ribotype of *A. magna* (Am1, 6067 reads, 24%) (Figure 3a,b). All major ribotypes of hexaploid *Avena* species are A-related, as are those of AC-genome species. The C-genome marker sequences remained only in a minor fraction (Figure 3a,b). They are related to the Cv-genome sequences of *A. ventricosa* but have accumulated many changes (Figure 3a,b). The ribotype tree shows the distinction of D-genome ribotypes (PP = 0.78, BS = 71), the separation of the main ribotypes of *A. canariensis* (PP = 0.88, BS = 98) and *A. atlantica* (PP = 0.86, BS = 73) (Figure 4).

In addition, the strongly supported clade comprises the C-genome sequences of *A. ventricosa* and polyploids (PP = 1, BS = 100) (Figure 4). *Avena ventricosa*, in turn, occupies its own place in the C-genome clade (PP = 0.98, BS = 98), while C-genome-related sequences of other species form separate subclades (Figure 4). In the tree, the number of reads in the C-genome fraction varies from 53 to 31.

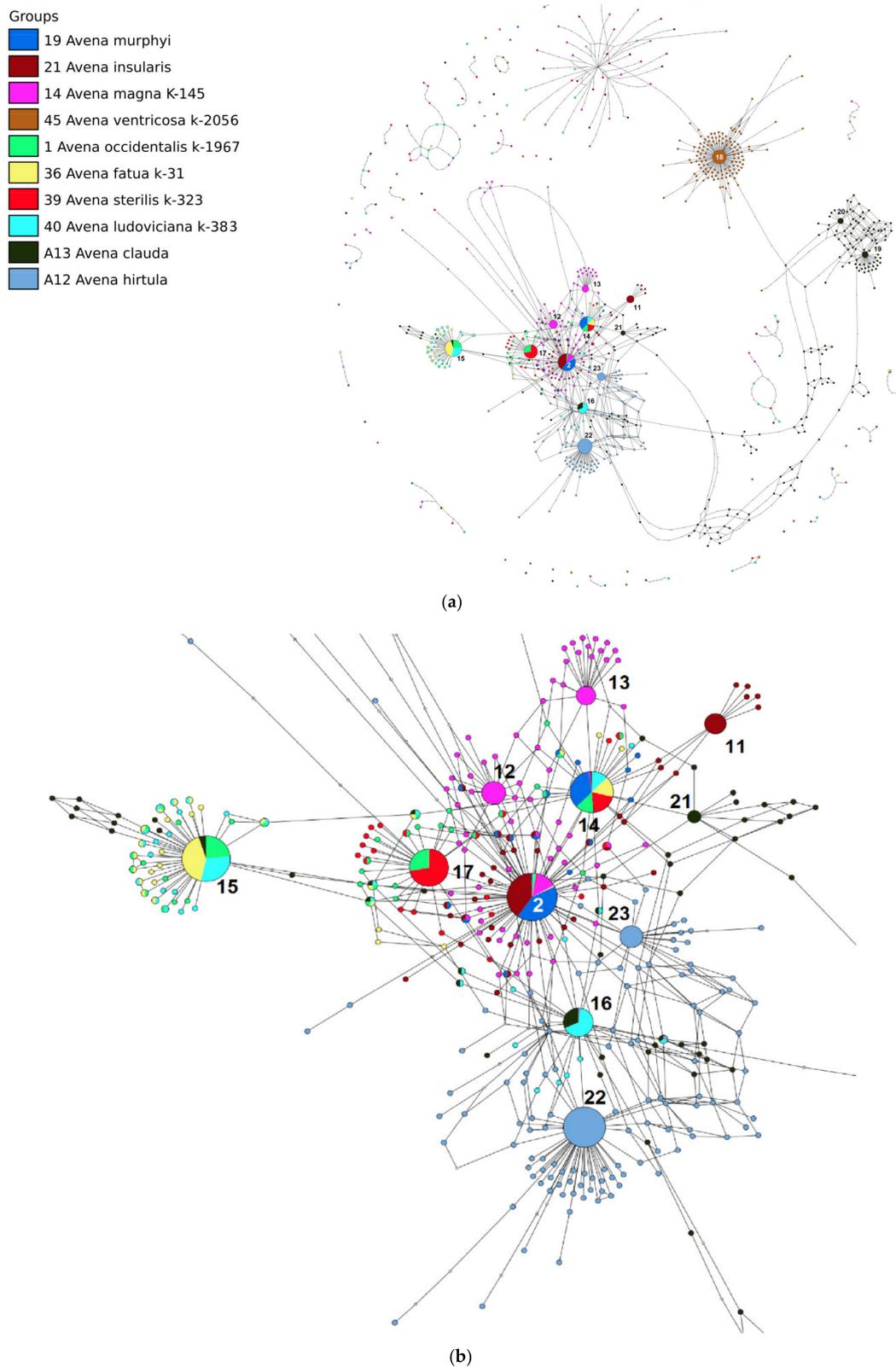
The third network shows the relationships between the ACD-genome hexaploids, *A. clauda*—a paleohomoploid species with the A- and C-genomes, and *A. hirtula*, the As-genome species.

The main ribotype of *A. clauda* (Cc1A, 5320 reads, 13%), together with one of the major ribotypes (Cc1B, 2695 reads, 7%), formed the C-genome ribotype network (26, b), but is not related to the C-genome-related sequences of the AC and ACD-genome species (Figure 5a,b). Meanwhile, the ribotypes of *A. clauda* CC2A–CC2C fell within the A-genome-related group [27], and some of them are shared with the A-genome ribotypes of polyploids (Figure 5a,b). *Avena clauda* ribotype CC2A (3195 reads, 8%) is common with *A. ludoviciana*, in which it is one of the major ribotypes (Ald, 3811 reads, 24%). The ribotype CC2B of *A. clauda* (1777 reads, 4%) is species-specific, and CC2C (1305 reads, 3%) is shared with the supposed the D-genome ribotype—the main ribotype of *A. fatua*, *A. ludoviciana*, and *A. occidentalis* (Figure 5a,b). We need to notice that possible the D-genome ribotype is common with a minor fraction of *A. hirtula* sequences (111 reads). The minor ribotype fraction of *A. hirtula* (579 reads) may be common with the As2 ribotype (Figure 5a,b). The ribotype tree shows the distinction of C-genome-related ribotypes (PP = 1, BS = 100, Figure 6).

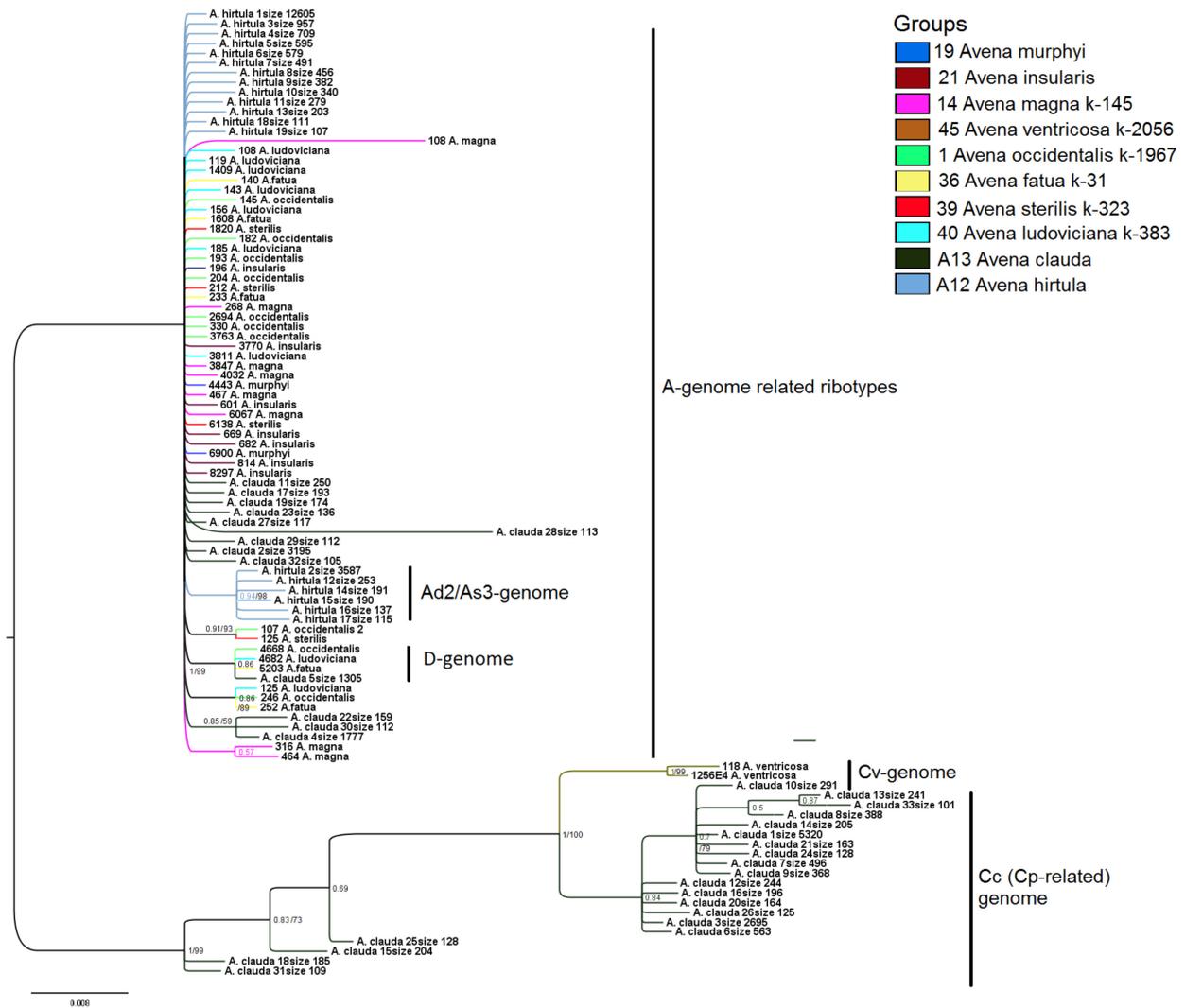
The Cv-genome ribotypes on the tree form a clade within those of the C-genome group (PP = 1, BS = 99) that also contained the C-genome ribotypes of *A. clauda* (Figure 6). The A-genome-related ribotypes form separate clades that consist of the second major ribotype of *A. hirtula* with allies (PP = 0.94, BS = 0.98); the D-genome ribotypes with one of *A. clauda* (PP = 0.86, BS = 99); minor ribotypes of *A. sterilis* and *A. occidentalis* (PP = 0.91, BS = 93); minor ribotypes of *A. ludoviciana*, *A. fatua*, and *A. occidentalis* (PP = 0.86, BS = 89), and minor ribotypes of *A. magna* (PP = 0.57, BS unsupported).



**Figure 4.** Phylogenetic tree of the ribotypes of wild hexaploid *Avena* species and their putative parental species. The first index on the branch is the posterior probability in Bayesian inference, and the second is the bootstrap index obtained by the Maximum Likelihood algorithm. When only one index is shown on the branch, it is the posterior probability. Numbers before the species name indicate the number of reads per rDNA pool.



**Figure 5.** (a) Ribotype network of wild hexaploid *Avena* species, the related tetraploids with AC-genome, and diploids *A. clauda* and *A. hirtula*. The radius of the circles on the ribotype network is proportional to the percentage of reads for each ribotype. Major ribotypes are larger than others (more than 1000 reads per rDNA pool) and marked with numbers. (b) Ribotype network of wild hexaploid *Avena* species, the related tetraploids with AC-genome, and diploids *A. clauda* and *A. hirtula*. More detailed picture of the major ribotypes.



**Figure 6.** Phylogenetic tree of the ribotypes of wild hexaploid *Avena* species, the related tetraploids with the AC-genome, and the diploids *A. clauda* and *A. hirtula*. The first index on the branch is the posterior probability in Bayesian inference, the second is the bootstrap index obtained by Maximum Likelihood algorithm. When only one index is shown on the branch it is the posterior probability. Numbers before the species name indicate number of the reads per rDNA pool. In the case of two species, *A. clauda* and *A. hirtula* number of reads per rDNA pool is placed after the word “size”.

### 3. Discussion

One of the main evolutionary pathways in the angiosperms and, in particular, in the grasses is allopolyploidization, which leads to new combinations of morphological features and adaptation to new environmental conditions. Then, after a series of massive chromosome rearrangements, a new chromosome set with the characteristics of a classic diploid may be established. Thus, for a complete understanding of the evolution in the active hybridizing group of some grass taxa, it is necessary to consider the chromosome set evolution against the background of the marker gene changes.

Previous research has found that two main groups of *Avena* chromosome sets—the A and C genomes—probably diverged from the common ancestor from 5 to 25 MYA, according to various estimates [32,33]. The A-genome also produced two variants, B and D, during its evolution [3,7,9–11]. Some recent studies placed the D-variant of the genome instead of the A-variant in previously defined AC-genome tetraploids [15,18,34], but our NGS analysis of marker sequences showed the presence of different variants of the A-genome not directly related to the putative D-genome in ACD-hexaploids. C-genome

oats are characterized by serious differences in their ITS sequences [35], and C-genome tetraploid *A. macrostachya* is probably the most primitive species according to ITS sequences and karyological analysis [26,35]. Our NGS studies showed some other differences from previously obtained results, and in some cases, they clarified the hypotheses based on morphological and cytogenetic data.

Despite a significant susceptibility to homogenization, ITS sequences have been widely used in research for interspecific and, in some cases, intraspecific phylogenetic reconstructions for a rather long time, since the 1990s. “Due to their levels of variation, ITS frequently provide enough molecular markers suitable for evolutionary studies at the species level. These include topics such as the origin of polyploid taxa, hybridization, introgression, and, above all, phylogenetic inference (up to 40% sequence divergence in pair-wise comparisons between congeneric taxa reported in early studies)” [36].

Intraspecific rDNA polymorphism has long been constantly used to study introgression resulting from hybridization, including due to hypothetical horizontal transfer, as well as to build phylogenetic hypotheses [37–39], etc. Multiple nucleotide sequences of ITS (in this case, ITS2) obtained by the next-generation sequencing method were taken for the phylogenetic analysis of *Abies* [40], with a certain emphasis on the distinction of the related species. As one of the universal barcodes and very popular DNA markers, the ITS region is very popular for establishing the hybridization picture in the complicated subtribe Aveninae (Poeae, Poaceae) using chloroplast sequences (tpF–atpH, psbK–psbI, psbA–rps19–trnH, matK, trnL–trnF) as independent markers because of their mostly maternal inheritance [41,42]. Moreover, complex of these markers corroborates the division of hybrid complexes involving intersubtribal and even intertribal hybridizations [41]. In our analysis, we use the whole pool of rDNA marker sequences obtained via next-generation sequencing (NGS). The advantage of this method is that in the rDNA pool, the sequences of different parental taxa of the allopolyploid species are retained. These sequences usually cannot be amplified by direct sequencing because of nucleolar dominance *Avena canariensis* (a diploid with the Ac-genome) is endemic to the Canary Islands. It is thought to be the most ancient among the A-genome oats [15,43], having diverged 13–15 MYA. According to previous data, *A. canariensis* could be the A-genome donor to AC and ACD species [3], or only to *A. agadiriana*, which is supposed to be the AB-genome species [15]. Our results, on the contrary, indicate only a little possible participation of *A. canariensis* in the polyploid formation; the ribotype of the minor fraction of *A. canariensis* is shared with the As2-ribotype of *A. atlantica* [27]. The ribotype As2 of *A. atlantica* was probably inherited by AC-genome tetraploids.

The AB-genome tetraploids have the As1-ribotype in their genome (the main ribotype in *A. atlantica*). While As-genome-related ribotypes are also present in other diploids (*A. wiestii*, *A. hirtula*), only two major ribotypes of *A. atlantica*, As1 and As2, were detected in tetraploids and hexaploids. *Avena barbata* most often has two satellite chromosomes in its tetraploid karyotype; one of them is heterobrachial with a large satellite attached to the short arm of the chromosome, and the second chromosome is strongly heterobrachial with a small satellite [44]. Such a pattern of satellite chromosomes can determine the number of major ribotypes in *A. barbata*, the second major ribotype is species-specific, and it is only 6% of the rDNA pool. However, and it is interesting, sometimes the third satellite chromosome occurs [44], and thus theoretically a new ribotype pattern can be observed. *Avena barbata* belongs to the AB-genome tetraploids; species-specific ribotypes can be variants of the B-genome. Two short awns on the lemma apex of *A. barbata* can show us the relationship of this species with primary diploids. The second AB-genome species, *A. vaviloviana*, has three major ribotypes; two of them are shared with minor ribotypes of *A. barbata*. The chromosomes of *A. vaviloviana* are prone to translocations, including those in satellite regions [15]. Thus, the increasing number of major ribotypes can be connected with the translocations. The second and third major ribotypes (14% and 5%, respectively) are related to the A-genome. They can be regarded as the species-specific variants of the B-genome sequences. *Avena vaviloviana* was at first thought to be a member of *A. strigosa* Schreb.

affinity, a subspecies of *A. strigosa* [45,46]. Their morphological resemblance may reflect the participation of the As-genome in this species.

The ribotype pattern in *A. agadiriana* differs from that of other AB-genome oats. *Avena agadiriana* has a special karyotype that is not found in any other tetraploids; in particular, it has two pairs of chromosomes with very small satellites and drastically asymmetrical centromeres [47]. In addition, early flowering forms of this species have three pairs of satellites on their chromosomes instead of two [48]. Nevertheless, its karyotype was designated as AB [8,18,34,49,50]. *Avena wiestii* and *A. canariensis* were supposed to be the ancestors of *A. agadiriana* by previous studies [7,8,34]. The results of FISH analysis of the karyotype of *A. agadiriana*, surprisingly, suggested determining its chromosome set as DD tetraploid instead of AB [15]. Our data, on the other hand, did not show a clear relationship of ribotypes of *A. agadiriana* and D-genome ribotypes of ACD-hexaploids. Instead, *A. agadiriana* has a species-specific main ribotype (Figure 1a,b) and two major ribotypes, one of which is shared with *A. atlantica* and *A. magna* (those among their minor ribotypes). Only the fourth of the ribotypes of *A. agadiriana* is shared with some other species as their major ribotype; it belongs to As2. The main ribotype of *A. agadiriana*, along with its different major ribotypes, can be derived from the A-genome and thus represent B-variants (specifically B1, B2, and B3). According to NGS data, *A. agadiriana* ribotype constitution may be described as BA instead of AB-variant as in *A. vaviloviana* and *A. barbata* because the main ribotype possibly represents B-variant, a derivative of ancestral A-genome. This special ribotype pattern may reflect the peculiar chromosome set of *A. agadiriana*. Results of the analysis of NGS data also confirmed the previous morphological observations: *A. agadiriana* resembles *A. barbata* in inflorescence characters [47]. Our data do not show the close relationship of *A. agadiriana* with *A. canariensis* (Ac-genome); it is related to As-genome of *A. atlantica* as well as to other AB-genome species.

*Avena magna*, *A. murphyi*, and *A. insularis* probably have the AC-genome constitution, according to our analysis. Previously, the AC-karyotype in these species was determined by various cytogenetic methods [3,11,13,51]. FISH-analysis with probes on the different genomes and chromosome assignments, on the contrary, detected the CD-genome variant in the AC-genome oats [15,18,19]. Similar results were presented by chromosome assignment in the linkage map [52]. Our NGS data obtained by sequencing the whole rDNA pool showed different ribotype patterns in *A. magna*, *A. murphyi*, and *A. insularis* species, though all these oats have ribotypes inherited from the A-genome of diploids. *Avena magna* has two species-specific major ribotypes as well as one As2-ribotype common with *A. atlantica* (Figure 1a,b). *Avena magna* has three satellite chromosomes and also the A-genome chromosomes, which are more similar to than those of hexaploids than of oats with the Ac-genome [53,54]. Cytogenetic studies also did not support the presence of a B-genome in *A. magna*. Specific major ribotypes in *A. magna* are very closely related to the A-genome ribotype variant (Table 2) and are not shared with the hexaploids, which have specific ribotypes. Thus, the main ribotype of *A. magna* can represent its peculiar genome that can pair with D-related probes, as revealed in the cytogenetic study by Tomaszewska et al. [15], but also with the *A. magna* genomic set, which retained possible ancestral sequences. The C-genome ribotypes in all AC-genome species of oats remained only in minor fraction (Figures 1 and 3a,b). There is a main network in the picture that contains the A-genome-related ribotypes (Figure 3a,b). The C-genome ribotypes, are related to the ribotypes of *A. ventricosa* as we see in Figure 3a,b, but have not any common ribotypes with it. Possibly, the C-genome-derived sequences in the allopolyploid genomes of tetra- and hexaploid species were drastically transformed through the post-hybridization processes. The elimination of the C-genome sequences in rDNA pool of allopolyploid species probably occurred because of significant differences between the sequences of two genome types [55].

From a morphological point of view, *A. murphyi* (AC) is close to *A. magna* (AC), and *A. insularis* (AC) is intermediate between *A. murphyi* and hexaploid *A. sterilis* (ACD) [11]. *Avena murphyi* and *A. insularis* are characterized by two denticles on the lemma apex [11]. The most represented ribotype in the rDNA pool of *A. murphyi* and *A. insularis* is As2

(Figures 1a,b and 3a,b, Table 2). As in *A. magna*, this fact may point to the participation of *A. atlantica* (or some extinct closely related diploid species) in the formation of a tetraploid. We previously assumed that only *A. atlantica* has two specific ribotypes, As1 and As2 [27], that were retained in the polyploid genome set (Figures 1a,b and 3a,b), whereas other species with the As-genome, *A. wiestii* and *A. hirtula*, have their own peculiar ribotypes that are partially common with the Ad-genome species and not further inherited by polyploid species. Both *A. murphyi* and *A. insularis* have two pairs of satellite chromosomes [13,56]. This probably caused the presence of two major ribotypes in these species (Figures 1a,b and 3a,b). The second major ribotype of *A. insularis* is species-specific, and that of *A. murphyi* is common with minor ribotypes of *A. magna*, *A. agadiriana*, and *A. insularis*. While FISH-analysis with genome-specific probes detected the CD-genome structure of *A. insularis* and *A. murphyi* [15] our data showed a closer relationship of these species and the A-genome species and a drastic elimination of the C-genome sequences from their rDNA. Furthermore, the As-genome of *A. insularis* and *A. murphyi* (their main ribotype) is not present among the ribotypes of hexaploids that can be attributed to the D-genome (Figure 3a,b). The second most represented ribotype in *A. murphyi* (variant Amp) could be A-genome ribotype inherited by the ACD-genome hexaploids (Figure 3a,b).

The ACD-genome hexaploid group contains the most widespread oat species, *A. fatua*. The D-genome is thought to be a derivative of the A-genome [11,20,21]. In this group, the A-genome comprises two pairs of satellite chromosomes (one pair with a large satellite and the other with a small satellite), and the C and D-genomes each have one pair of satellite chromosomes with small satellite [57,58]. It is interesting that at first their karyotype constitution was determined as ABC [59]. Recent studies supported the ACD-genome variant in hexaploids [15] and suggested different hypotheses about their origin [19]. For example, SSR-FISH analysis of the hexaploid species *A. byzantina* and *A. sativa* showed *A. insularis* as a tetraploid species closest to hexaploid ones [60]. FISH-analysis with genome-specific probes revealed *A. strigosa* as the A-genome progenitor of the ACD-oats while *A. insularis* is thought to be the CD-genome donor of *A. sterilis* which is, in turn, the ancestral of all hexaploids [15]. Our data clearly supported the ACD-genome constitution for the studied hexaploid species (Figure 3a,b) although the pattern of relationships between tetra- and hexaploids differs from the assumptions based on FISH data [15]. The most frequent ribotype in the species *A. occidentalis*, *A. fatua*, and *A. ludoviciana* is specific variant that does not occur in any other diploid or polyploid species except paleopolyploid *A. clauda* (see below). This variant can be described as the D-subgenome. However, the main ribotype in hexaploid *A. sterilis* differs from the most represented ribotypes of other hexaploids. It is common with the second major ribotype of *A. occidentalis*. This ribotype is connected to the A-subgenome (Figure 3a,b) and can be named Ast or D' (Table 2). *Avena sterilis* was thought to be an ancestral species for the hexaploids [45,61]. On the contrary, specific major ribotypes of *A. sterilis* and *A. occidentalis* may suggest the separate origin of these species, independent from other ACD-genome oats. The second of the major ribotypes of *A. fatua* is shared with *A. murphyi*, its second major ribotype, which may be named as Amp (Figure 3a,b, Table 2). This contradicts the previous hypotheses of *A. canariensis* as the Ac-genome progenitor of all hexaploids [3,11], and also of *A. magna* as the parental taxon for the hexaploids [62]. Instead, *A. murphyi* could form its specific ribotype in its polyploid evolution and then participate in the formation of *A. fatua* and other hexaploids. *Avena ludoviciana* is treated as a relative of *A. sterilis* by morphological criteria [11]. It has the species-specific second major ribotype along with possible D-genome-related and amp ribotypes (Figure 3a,b, Table 2). The specific ribotype Ald belongs to the A-genome group. According to the ribotype pattern, *A. ludoviciana* is not closely related to *A. sterilis*. Based on our NGS sequence analysis, *A. ludoviciana*, *A. occidentalis*, and *A. fatua* form the natural group of affinity, where *A. fatua* has fewer variants of ribotypes than others. Thus, *A. fatua* probably could have gone through the stages of hybrid stabilization or evolved independently from other hexaploid species. Regarding the results of the NGS analysis, the C-genome rDNA could derive from *A. ventricosa* or some extinct the Cv-genome species

(Figures 3a,b and 4), though its sequences have been probably greatly transformed by post-hybridization processes such as conversion. They were retained only in minor fraction being strongly different from the prevailing sequences of the A-genome. It supports the previous results of ITS analysis [63]. All sequences of hexaploid oats that were obtained by Sanger method clearly group with A-genome taxa [63]. This fact can indicate the elimination of the distantly related sequences from the pool rDNA of complex hybrid (see also [64], IGS data) and probably inactivating of some sequences for establishing the genome balance [55,65,66].

We also need to mention that the As-genome diploid oat, *A. hirtula* (or its ancestor), could participate in the formation of polyploid species. Major ribotypes of *A. hirtula* are not shared with any of the polyploids, but some sequences of its minor fraction are shared with *A. ludoviciana*—its second major ribotype (Figure 5a,b). In addition, one ribotype of the minor fraction of *A. hirtula*, in addition, is identical with As2-ribotype that is common for *A. murphyi*, *A. magna*, and *A. insularis*. Previous data placed different marker sequences of *A. hirtula* near those of *A. strigosa* [67] or in a clade with *A. atlantica* and *A. lusitanica* (Tab.Morais) B.R.Baum (= *A. barbata*), sister to clade including the hexaploids [7]. As was supposed in previous studies, *A. hirtula* probably evolved from *A. atlantica* [11,15] and thus it could keep ancestral ribotype variants in its minor fraction of rDNA.

One of the most interesting questions is the possible participation of the paleopolyploid (now diploid) species *A. clauda* in the formation of hexaploids. Phylogenetic data obtained from low copy sequences showed that *A. clauda* was more closely related to the hexaploids than *A. ventricosa* [7]. However, our data indicated that C-genome rDNA in tetra- and hexaploid oats was obtained from *A. ventricosa*. *Avena clauda*, being a putative ancient hybrid [26,27], in turn had A-genome-related ribotypes in its rDNA pool that could further form the D-genome rDNA in *A. occidentalis*, *A. fatua*, and *A. ludoviciana*. The possible belonging of the major ribotypes of *A. occidentalis*, *A. fatua*, and *A. ludoviciana* to the D-genome is proved by the fact that these sequences do not occur in any other studied oats except hexaploids. However, chromosome constitution of *A. clauda* characterizes this species as the C-genome bearer [46]. Four chromosomes Therefore, *A. clauda* could be the ancient hybrid between A- and C-genome oats from which the A-genome chromosomes were eliminated but some rDNA sequences have been retained after the post-hybridization transformation. In addition, one of the major A-genome-related ribotypes of *A. clauda* could be inherited by *A. ludoviciana* (Figure 5a,b). From the morphological point of view, *A. clauda* propagates by separate caryopses instead of the whole spikelets and has very unequal glumes; so, it may be one of the most primitive oats [3,56]. Probably, *A. clauda* or some extinct relative of this species participated in the formation of at least some hexaploids with the D-genomes.

#### 4. Conclusions

NGS data, as we see, can be a convenient tool for phylogenetic reconstruction of the evolution of hybrid species. A rather small but stable quantity of different nucleotide positions, combined with the data obtained by cytogenetic methods, can provide us with detailed information about the allopolyploidization patterns in the genera of grasses. Our results supported some earlier hypotheses about the origin of polyploid *Avena* species. We assume that tetraploid oats that were previously defined as AC-genome species actually have this genomic constitution according to the marker ITS1 sequences. The D-genome sequences were not found in the AC-genome tetraploid oats that were related to those with the ACD-genome. The A-genome-related sequences were possibly inherited from the As-genome species *A. atlantica*, while the D-genome-related sequences were formed already in hexaploid oats or were taken from an unknown ancestor related to *A. clauda*. The A-genome sequences in the ACD-genome *Avena* species were probably inherited from *A. murphyi* (AC). Alternatively, the AC-genome-related ITS1 sequences could persist in the CD-genome oats because of translocation events detected by Yan et al. [59] when the A-genome chromosomes were transformed into the D-genome ones after a series of introgressive hybridizations.

## 5. Materials and Methods

We took into analysis 13 wild species of *Avena* belonging to three genome groups: AB (*A. agadiriana*, *A. barbata*, and *A. vaviloviana*), AC (*A. insularis*, *A. magna*, and *A. murphyi*), and ACD (*A. fatua*, *A. ludoviciana*, *A. occidentalis*, and *A. sterilis*). In addition, we studied putative diploid progenitors of the tetraploids—*A. atlantica* (the As-genome), *A. canariensis* (Ac), and *A. ventricosa* (Cv). All material was taken from the Federal Research Center N. I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR). A summary of the studied species is presented in Table 1.

Genomic DNA was extracted from dried leaf and seed material using a Qiagen Plant Mini Kit (Qiagen Inc., Hilden, Germany). For NGS analysis, we took 18S–ITS1–5.8S rDNA region. The fragments were amplified and sequenced at the Center for Shared Use “Genomic Technologies, Proteomics, and Cell Biology” of the All-Russian Research Institute of Agricultural Microbiology on an Illumina Platform MiSeq. PCR was carried out in 15 µL of the reaction mixture containing 0.5–1 unit of activity of Q5<sup>®</sup> High-Fidelity DNA Polymerase (NEB, Ipswich, MA, USA), 5 pM of forward and reverse primers, 10 ng of DNA template, and 2 nM of each dNTP (Life Technologies, ThermoScientific, Waltham, MA, USA). It was amplified under the following conditions: initial denaturation at 94 °C for 1 min, followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final elongation 72 °C for 5 min using ITS 1P [68] and ITS 2 [69] primers. PCR products were purified according to the Illumina recommended method using AMPureXP (Beckman Coulter, Indianapolis, IN, USA). Then the libraries were prepared according to the manufacturer’s MiSeq Reagent Kit Preparation Guide (Illumina) (<http://web.uri.edu/gsc/files/16s-metagenomic-library-prep-guide-15044223-b.pdf> (accessed on 11 May 2020)). They were sequenced on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA) using a MiSeq<sup>®</sup> ReagentKit v3 (600 cycles) with double-sided reading (2 × 300 n) following the manufacturer’s instructions. The sequences were trimmed with Trimmomatic [70], included in Unipro Ugene [71] using the following parameters: PE reads, sliding window trimming with size 4, quality threshold 12, and minimal read length 130. Further, paired marker sequences were combined, dereplicated, and sorted into the ribotypes with the aid of vsearch 2.7.1 [72]. Then the sequences that represent ribotypes with certain frequency in the whole genome pool were analyzed by TCS 1.21 [73] and visualized in TCSBU [74]. The resulting network was built according to the statistical parsimony algorithm and shows possible hybridization events in the species complex. The threshold for this analysis was 10 reads per the whole genome pool. Additionally, we built phylogenetic trees of the ribotypes by Bayesian inference and maximum likelihood method. The threshold for tree inference was 30 reads per the rDNA pool, except the dataset with hexaploid species and *Avena clauda*, here we took the threshold 100 reads per the rDNA pool. In the first case we used Mr. Bayes 3.2.2 [75] and in the second case, iqtree 1.6.12 (<http://www.iqtree.org/>, accessed on 1 March 2023) using the GTR+G model previously estimated by MEGA XI [76]. Bayesian analysis was conducted with 5–8 million of generations, sampling trees every 100 generations. and the first 25% trees were discarded as burn-in. The ML search was performed under the fast bootstrap option, 1000 generations. The obtained trees were edited in FigTree 1.4.3 (<http://tree.bio.ed.ac.uk/>, accessed on 1 March 2023). The resulting tree combines Bayesian and ML data. The first index is posterior probability, and the second is bootstrap index.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/d15060717/s1>, Table S1. Aligned ribotype sequences of the tetraploid oats and their probable parental species obtained via NGS. Table S2. Aligned ribotype sequences of the hexaploid oats and their probable parental species obtained via NGS. Table S3. Aligned ribotype sequences of the hexaploid oats, their tetraploid relatives with AC-genome the and diploids *A. clauda* and the *A. hirtula*.

**Author Contributions:** A.A.G. and N.N.N. carried out the experiments. A.A.G., N.N.N. and A.V.R. analyzed the data. I.G.L. and E.V.B. provided seed material. A.A.G., N.N.N. and A.V.R. wrote the manuscript. V.S.S. thoroughly corrected and edited the text. All authors have read and agreed to the published version of the manuscript.

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