Phylogenetic relationships in the genus *Avena* based on the nuclear *Pgk1* gene

Yuanying Peng[1], Pingping Zhou[1,2], Jun Zhao[1], Junzhuo Li[1], Shikui Lai[1], Nicholas A. Tinker[3], Shu Liao[1], Honghai Yan[1,2*]

[1]Triticeae Research Institute, Sichuan Agricultural University, Chengdu, People's Republic of China

[2]Collaborative Innovation Center of Tissue Repair Material of Sichuan Province, China West Normal University, Nanchong, People's Republic of China

[3]Ottawa Research and Development Centre, Agriculture and Agri-Food Canada, Ottawa, Canada

*Corresponding author

E-mail: Honghai_yan@outlook.com

Abstract

The phylogenetic relationships among 76 *Avena* taxa, representing 14 diploids, eight tetraploids, and four hexaploids were investigated by using the nuclear plastid 3-phosphoglycerate kinase gene(*Pgk1*). A significant deletion (131 bp) was detected in all the C genome homoeologues which reconfirmed a major structural divergence between the A and C genomes. Phylogenetic analysis indicated the C_p genome is more closely related to the polyploid species than is the C_v genome. Two haplotypes of *Pgk1* gene were obtained from most of the AB genome tetraploids. Both types of the *barbata* group showed a close relationship with the A_s genome diploid species, supporting the hypothesis that both the A and B genomes are derived from an A_s genome. Two haplotypes were also detected in *A. agadiriana*, which showed close relationships with the A_s genome diploid and the A_c genome diploid, respectively, emphasizing the important role of the A_c genome in the evolution of *A. agadiriana*. Three homoeologues of the*Pgk1* gene were detected in five hexaploid accessions. The homoeologues that might represent the D genome were tightly clustered with the tetraploids *A. marrocana* and *A. murphyi*, but did not show a close relationship with any extant diploid species.

Introduction

The genus *Avena* L. belongs to the tribe Aveneae of the grass family (Poaceae). It contains approximately 30 species [1-4] reflecting a wide range of morphological and ecological diversity over the temperate and subtropical regions [5]. The evolutionary history of *Avena* species has been discussed for decades, and

remains a matter of debate despite considerable research effort in this field. Cytologically, three ploidy levels are recognized in the genus *Avena*: diploid, tetraploid, and hexaploid, with a base number of seven chromosomes [6, 7]. The diploids are divided clearly into two distinct lineages with the A and C genomes. All hexaploid species share the same genomic constitution of ACD, corroborated by fertile interspecific crosses among each other, as well as by their similar genome sizes [8]. With less certainty, the tetraploids have been designated as AB or AA, AC or DC, and CC genomes [9]. It is noteworthy that the B and D genomes within the polyploid species have not been identified in any extant diploid species. There are three C genome diploid species, which have been grouped into two genome types (C_p and C_v) according to their karyotypes [10]. Both types show a high degree of chromosome affinity to the polyploid C genome [9-14], but none have been undisputedly identified as the C genome progenitor of the polyploids.

The A genome origin of polyploid oats has also been under intense scrutiny. However, there is no conclusive evidence regarding which the A genome diploid contributed to the polyploid oats. There are up to 12 species designated as A genome diploids. These species have been further subdivided into five sub-types of A_c, A_d, A_l, A_p and A_s genomes, according to their karyotypes [6, 7]. Most research based on karyotype comparisons [6, 15], in situ hybridization [11, 16-18], as well as the alignments of nuclear genes [13, 14] suggest that one of the A_s genome species may be the A genome donor of polyploid oats. Alternatively, some studies have

proposed the A_c genome diploid *A. canariensis* [19], or the A_l genome diploid *A. longiglumis* [9, 12] as the most likely A genome donor.

The absence of diploids with the B and D genomes complicates the B and D genome donor identification. It is generally accepted that both B and D genomes are derived from A genomes, due to the high homology between the B and A genomes [11, 20], as well as between the D and A genomes [16, 19, 21]. Our recent study based on high-density genotyping-by-sequencing (GBS) markers [9] provided strong evidence that the three tetraploid species formerly designated as AC genomes are much closer to the C and D genomes of the hexaploids than they are to the hexaploid A genome. These findings suggest that the hexaploid D genome exists in the extant tetraploids. However, no extant diploid species, even the A_c genome diploid *A. canariensis,* which was considered as the most likely D genome progenitor based on direct evidence from morphological features [22] and indirect evidence from fluorescent in situ hybridization (FISH) [18], showed enough similarity to the D genome of tetraploid and hexaploid oats to warrant consideration as a direct D genome progenitor.

In the case of the B genome, an initial study of chromosome pairing of hybrids between the AB genome tetraploids and the A_s genome diploids suggested that the B genome arose from the A_s genome through autoploidization [23]. Recently, another GBS study [19] showed that the AB genome tetraploid species fell into a tight cluster with A_s genome diploids, also supporting the hypothesis that the B genome arose through minor divergence following autoploidization. However,

other evidence from C-banding [24], FISH [17], RAPD markers [25], and DNA sequence alignment [14] has indicated a clear distinction between A and B genomes, suggesting an allotetraploid origin of the AB genome tetraploid species. The most probable A genome progenitor of the AB genome tetraploids is assumed to be an A_s genome diploid species, while the B genome of these species remains controversial.

Single or low copy nuclear genes are widely used in phylogenetic analyses due to their bi-parental inheritance and to the informativeness of mutations. Such studies have successfully revealed multiple polyploid origins, and clarified hybridization events in a variety of plant families [26, 27]. In a previous study [14], we investigated the relationships among *Avena* species by sequencing the single-copy nuclear acetyl-coA carboxylase gene (*Acc1*). The results provided some useful clues to the relationships of *Avena* species.

The *Pgk1* gene, which encodes the plastid 3-phosphoglyceratekinase, is another nuclear gene that has been widely used to reveal the evolutionary history of the *Triticum/Aegilops* complex due to its single copy status per diploid chromosome in grass [26, 28, 29]. The *Pgk1* gene is now considered to be superior to the *Acc1* gene in phylogenetic analysis,since it has more parsimony informative sites than the *Acc1* gene [26, 29]. In the present study, we sequenced cloned *Pgk1*gene copies from 76 accessions representing the majority of *Avena* species, in an attempt to further clarify evolutionary events in this important genus.

Materials and Methods

Plant materials

A total of 76 accessions from26 *Avena* species were investigated to represent the geographic range of six sections in *Avena*, together with one accession from *Trisetopsis turgidula* as a functional outgroup (Table 1). All seeds were provided by Plant Gene Resources of Canada (PGRC) or the National Small Grains Collection, Agriculture Research Service, United States Department of Agriculture (USDA, ARS) with the exception of the three accessions of *A. insularis*, which were kindly provided by Dr. Rick Jellen, Brigham Young University, Provo, UT, USA. The species *A. atherantha*, *A. hybrida*, *A. matritensis* and *A. trichophylla* described in Baum's [1] monograph and *A. prostrata* described by Ladizinsky [30] were not included due to a lack of viable material.

Table 1. List of materials used in the present study including species, haplomes, accession number, origin, abbreviation displayed in MJ network, and the sequence number in Genbank (https://www.ncbi.nlm.nih.gov).

Taxa	Haplomes	Accession Number	Origin*	Abbrev-iation	Genbank Accession
Section *Ventricosa*					
A. clauda Dur.	C_p	CN 19242	Turkey	CLA1_1	KU888786
		CN 21378	Greece	CLA2_1	KU888787
		CN 21388	Algeria	CLA3_1	KU888804
		CN 24695	Turkey	CLA4_1	KU888784
A. eriantha Dur. (syn *A. pilosa* Bieb.)	C_p	CIav 9050	United Kingdom	ERI1_1	KU888785
		PI 367381	Madrid, Spain	ERI2_1	KU888805
A. ventricosa Balansa ex Coss.	C_v	CN 21405	Algeria	VEN1_1	KU888806
		CN 39706	Azerbaijan	VEN2_1	KU888807
Section *Agraria*					
A. brevis Roth	A_s	CIav 1783	German	BRE1_1	KU888707

		CIav 9113	Europe	BRE2_1	KU888718
		PI 258545	Portugal	BRE3_1	KU888710
A. hispanica Ard.	A_s	CN 25676	Portugal	HIS1_1	KU888714
		CN 25727	Portugal	HIS2_1	KU888711
		CN 25766	Portugal	HIS3_1	KU888709
		CN 25778	Portugal	HIS4_1	KU888712
A. nuda L.	A_s	PI 401795	Netherlands	NUD1_1	KU888734
A. strigose Schreb.	A_s	PI 83722	Australia	STR1_1	KU888719
		PI 158246	Lugo, Spain	STR2_1	KU888713
		CIav 9066	Ontario, Canada	STR3_1	KU888708
Section *Tenuicarpa*					
A. agadiriana Baum & Fedak	AB	CN 25837	Africa: Morocco	AGA1_1	KU888753
				AGA1_2	KU888774
		CN 25854	Africa: Morocco	AGA2_1	KU888777
				AGA2_2	KU888754
		CN 25856	Africa: Morocco	AGA3_1	KU888776
				AGA3_2	KU888751
		CN 25863	Africa: Morocco	AGA4_1	KU888775
		CN 25869	Africa: Morocco	AGA5_1	KU888752
				AGA5_2	KU888778
A. atlantica Baum & Fedak	A_s	CN 25849	Africa: Morocco	ATL1_1	KU888757
		CN 25859	Africa: Morocco	ATL2_1	KU888756
		CN 25864	Africa: Morocco	ATL3_1	KU888739
		CN 25887	Africa: Morocco	ATL4_1	KU888737
		CN 25897	Africa: Morocco	ATL5_1	KU888736
A. barbata Pott ex Link	AB	PI 296229	Northern, Israel	BAR1_1	KU888723
		PI 337802	Izmir, Turkey	BAR2_1	KU888722
				BAR2_2	KU888732
		PI 337826	Greece	BAR3_1	KU888720
		PI 282723	Northern, Israel	BAR4_1	KU888729
		PI 337731	Macedonia, Greece	BAR5_1	KU888731
		PI 367322	Beja, Portugal	BAR6_1	KU888730
A. canariensis Baum et al	A_c	CN 23017	Canary Islands	CAN1_1	KU888779
		CN 23029	Canary Islands	CAN2_1	KU888782
		CN 25442	Canary Islands	CAN3_1	KU888780
		CN 26172	Canary Islands	CAN4_1	KU888783
		CN 26195	Canary Islands	CAN5_1	KU888781
A. damascena Rajah & Baum	A_d	CN 19457	Syria	DAM1_1	KU888744
		CN 19458	Syria	DAM2_1	KU888745

		CN 19459	Syria	DAM3_1	KU888747
A. hirtula Lag.	A_s	CN 19530	Antalya, Turkey	HIR1_1	KU888738
		CN 19739	Algeria	HIR2_1	KU888762
		CN 21703	Morocco	HIR3_1	KU888717
A. longiglumis Dur.	A_l	CIav 9087	Oran, Algeria	LON1_1	KU888741
		CIav 9089	Libya	LON2_1	KU888749
		PI 367389	Setubal, Portugal	LON3_1	KU888750
A. lusitanica Baum	A_s	CN 25885	Morocco	LUS1_1	KU888746
		CN 25899	Morocco	LUS2_1	KU888748
		CN 26265	Portugal	LUS3_1	KU888742
		CN 26441	Spain	LUS4_1	KU888763
A. wiestii Steud.	A_s	PI 53626	Giza, Egypt	WIE1_1	KU888715
		CIav 9053	Ontario, Canada	WIE2_1	KU888716
Section *Ethiopica*					
A. abyssinica Hochst.	AB	PI 411163	Seraye, Eritrea	ABY1_1	KU888724
		PI 411173	Tigre, Ethiopia	ABY2_1	KU888740
				ABY2_2	KU888725
A. vaviloviana Mordv.	AB	PI 412761	Eritrea	VAV1_1	KU888743
				VAV1_2	KU888728
		PI 412766	Shewa, Ethiopia	VAV2_1	KU888726
				VAV2_2	KU888735
Section *Pachycarpa*					
A. insularis Ladiz.	AC(DC)	sn	Sicily, Italy	INS1_1	KU888794
				INS1_2	KU888705
		6-B-22	Sicily, Gela, Italy	INS2_1	KU888706
				INS2_2	KU888796
		INS-4	Sicily, Gela, Italy	INS3_1	KU888790
				INS3_2	KU888704
A. maroccana Grand. (syn. *A magna* Murphy et Terrell)	AC(DC)	CIav 8330	Morocco	MAR1_1	KU888773
				MAR1_2	KU888799
		CIav 8331	Khemisset, Morocco	MAR2_1	KU888721
				MAR2_2	KU888800
A. murphyi Ladiz.	AC(DC)	CN 21989	Spain	MUR1_1	KU888767
				MUR1_2	KU888802
		CN 25974	Morocco	MUR2_1	KU888769
				MUR2_2	KU888788
Section *Avena*					
A.fatua L.	ACD	PI 447299	Gansu, China	FAT1_1	KU888768
				FAT1_2	KU888795
		PI 544659	United States	FAT2_1	KU888764

				FAT2_2	KU888760
				FAT2_3	KU888798
A.occidentalis Dur.	ACD	CN 4547	Canary Islands, Spain	OCC1_1	KU888791
		CN 23036	Canary Islands, Spain	OCC2_1	KU888755
				OCC2_2	KU888803
				OCC2_3	KU888771
		CN 25942	Morocco	OCC3_1	KU888733
				OCC3_2	KU888789
				OCC3_3	KU888758
		CN 25956	Morocco	OCC4_1	KU888801
				OCC4_2	KU888772
A. sativa L.	ACD	PI 194896	Gonder, Ethiopia	SAT1_1	KU888727
				SAT1_2	KU888759
				SAT1_3	KU888793
		PI 258655	Russian Federation	SAT2_1	KU888797
				SAT2_2	KU888766
				SAT2_3	KU888761
A. sterilis L.	ACD	PI 411503	Alger, Algeria	STE1_1	KU888765
		PI 411656	Tigre, Ethiopia	STE2_1	KU888792
				STE2_2	KU888770
Outgroup *Trisetopsis turgidula* Röser & A. Wölk		PI 364343	Maseru, Lesotho		KU888808

* Origin represents the collection site of wild material where this information is available, otherwise it represents the earliest source for which information is available.

DNA isolation, cloning and sequencing

Genomic DNA was isolated from fresh leaves of single plants following a standard CTAB protocol [31]. *Pgk1* gene sequences were amplified by using a pair of *Pgk1*-specific primers, PGKF1 (5'-TCGTCCTAAGGGTGTTACTCCTAA-3') and PGKR1 (5'-ACCACCAGTTGAGATGTGGCTCAT-3') described by Huang et al. [28]. Polymerase

chain reactions (PCR) were carried out under cycling conditions reported previously [26]. After estimating the size by 1.0% agarose gel, PCR products were purified using the QIAquick gel extraction kit (QIAGEN Inc., USA). The purified products were cloned into the pMD19-T vector (Takara) following the manufacturer's instructions. Initially, 6-8 positive clones from each of four accessions from 4 diploid species,including *A. canariensis* (A_c), *A. longiglumis* (A_l), *A. strigosa* (A_s), and *A. clauda* (C_p), were sequenced to confirm that the *Pgk1* gene was present in *Avena* diploid species as a single copy. After confirming its single copy status in diploid species, 2-3 positive clones were selected and sequenced from each accession of the remaining diploid species. In order to isolate all possible homoeologous sequences in polyploid species, 4-6 positive clones from each accession of the tetraploid species and 5-10 positive clones from each accession of the hexaploid species were selected and sequenced. All the cloned PCR products were sequenced on both strands by a commercial company (Sangon Biotech Co., Ltd., Shanghai, China) based on Sanger sequencing technology.

Sequence alignment and phylogenetic analysis

The homology of sequences was verified usingthe BLAST program in NCBI. In order to reduce the matrix size of the dataset, redundant sequences were removed, keeping one representative sequence if several identical sequences were derived from the same accession. Sequences were aligned using ClustalW software with default parameters [32] followed by manual correction. Substitution saturation of *Pgk1* sequenceswasexamined using DAMBE version 5 [33] by calculating and plotting

pairwise rates of transitions and transversions against sequence divergence under the TN93 model. Phylogenetic trees were created by using Maximum parsimony (MP), and Bayesian inference (BI). MP analysis was performed on PAUP* 4.0b10 [34] using the heuristic search with 100 random addition sequence replicates and Tree Bisection-Reconnection (TBR) branch swapping algorithms. Bootstrapping with 1000 replicates was estimated to determine the robustness of formed branches [35]. Gaps in the sequence alignment were disregarded using the option 'gapmode=missing', which is consistent with an assumption that insertion/deletion events are an independent stochastic process from SNP substitutions. BI analysis was carried out by using MrBayes v3.2 [36]. The best-fit substitution model for BI analysis was GTR+Γ+I, which was determined by using MrModelTest v2.3 under Akaike information criteria (AIC) (http://www.ebc.uu.se/systzoo/staff/nylander.html). Four Markov chain Monte Carlo (MCMC) chains with default priors settings were run simultaneously. To ensure the two runs converged onto the stationary distribution, 6,000,000 generations were run to make the standard deviation of split frequencies fall below 0.01. Samples were taken every 100 generations. The first 25% samples from each run were discarded as the "burn-in". The 50% majority-rule consensus tree was constructed from the remaining trees. Posterior probability (PP) values were used to evaluate the statistical confidence of each node.

Network analysis

The median-joining (MJ) network [37] method has been demonstrated to be an effective method for assessing the relationship in closely related lineages [38],

and thus was applied in this study. As MJ algorithms are designed for non-recombining molecules [37], DNA recombination was test by using a pragmatic approach-Genetic Algorithm Recombination Detection (GARD), described by Pond et al. [39]. The test was carried out on a web-based interface for GARD at http://www.datamonkey.org/GARD/. Building upon this test, the intron data was used for MJ reconstruction due to the absence of recombination signal, while potential recombination signals were detected in the exon regions. The MJ network analyses was performed using the Network 4.6.1.4 program (Fluxus Technology Ltd, Clare, Suffolk, UK).

Results

Sequence analysis

A total of 237 clones were sequenced from 76 accessions of 26 *Avena* species. Following removal of the redundant sequences within each accession, 104 sequences were identified, including one from each of the 44 diploid accessions, 37 unique sequences from 22 tetraploids, and 23 from 10 hexaploids. Theoretically, 44 homoeologues should be isolated from 22 tetraploid accessions, and 30 single-copy homoeologues were expected from 10 hexaploid accessions. However, the full number of expected homoeologues were not isolated from every polyploid species for various potential reasons. In particular, within the AB genome tetraploid species *A. barbata*, only one copy was detected in five of its six accessions, whereas two very similar (only one site varied in exon 2) copies were detected in the sixth accession. This also happened in the hexaploid species *A. sterilis*, for which two accessions

196 provided only two homoeologues each. For these taxa, the missing genome type
197 might be detected by screening a larger number of positive clones, but it is also
198 possible that these accessions contain genomes of high similarity or autopolyploid
199 origin. Another possibility that cannot be ruled out within the polyploids is the loss
200 of one gene copy through homoeologous recombination or deletion.

201 All of the *Pgk1* gene sequences isolated in this study contain 5 exons and 4
202 introns, covering a total length from 1391 bp to 1527 bp, which is consistent with
203 previous studies of this gene in wheat [28]and *Kengyilia* [26]. The alignment of *Pgk1*
204 sequences including both exons and introns resulted in a matrix of 1539 nucleotide
205 positions, of which 11.6% (179/1539) were variable, and 10.1% (155/1539) were
206 parsimony informative. The nucleotide frequencies were 0.264 (A), 0.304 (T), 0.199
207 (C), and 0.232 (G). A significant (131-bp) insertion/deletion feature (Fig 1A) occurred
208 at position 968, whereby all non-C genome type sequences contained the inserted
209 (or non-deleted) region. Further analysis indicated that this region is likely an
210 inserted inverted repeat, which belongs to the MITE stowaway element. Its
211 secondary structure is shown in Fig 1B. This insertion/deletion event could be used
212 as a genetic marker for rapid diagnosis of *Avena* species containing the C genome.

Fig 1. *Pgk1* gene sequence analysis. (A) Partial alignment of the amplified *Pgk1* gene of *Avena* species (B) Secondary structure of the deletion sequence between the A and C genomes.

Phylogenetic analyses

The substitution plot for *Pgk1* (Fig 2) indicated that the *Pgk1* gene was not saturated and that it could be used for phylogenetic analysis. Phylogentic trees of 76 *Avena* accessions with the oat-like species *Trisetopsis turgidula* as outgroup were generated through maximum parsimony and Bayesian inference approaches on the non-redundant dataset. The parsimony analysis resulted in 80 equally parsimonious trees (consistency index (CI) =0.632, retention index (RI) =0.954). BI analysis inferred an almost identical tree topology as the MP analysis, so the MP results were selected to describe this study (Fig 3).

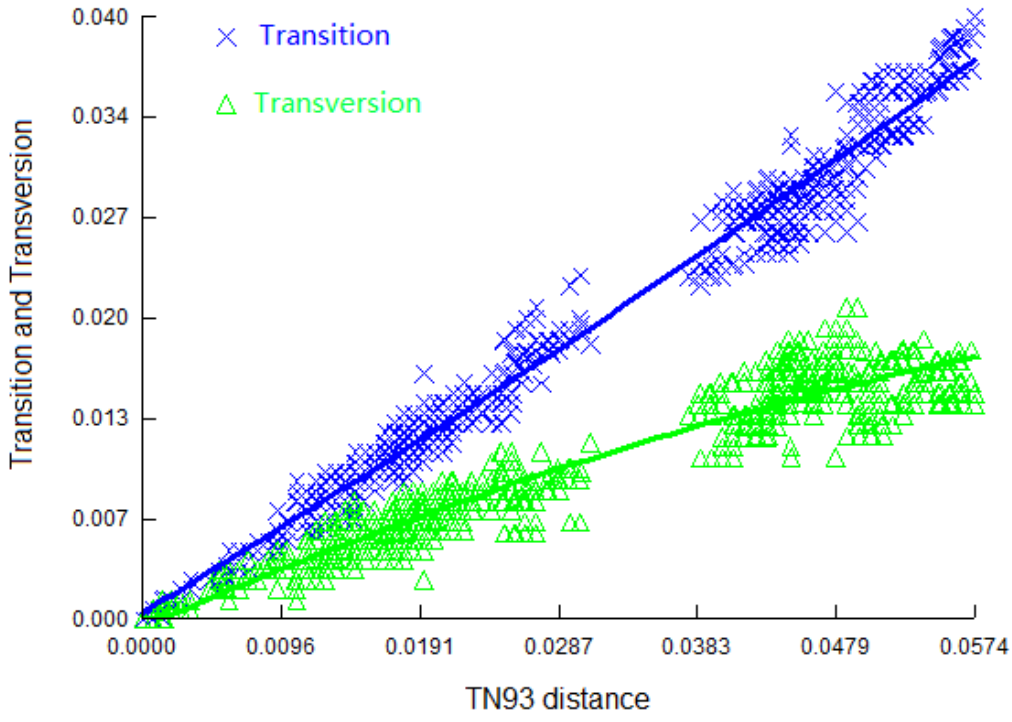


Fig 2. Saturation plot for transition and transversion of *Pgk1* gene sequences. The crosses are the number of transition events; the triangles are the number of transversion events. The x axis shows the genetic distance based on the TN93 model; the y axis is the proportion of transitions or tansversions, which was calculated by

using the number of transitions or transversions divided by the sequence length. The curves show the trends of the variance of transitions and transversions with the genetic distance increasing.

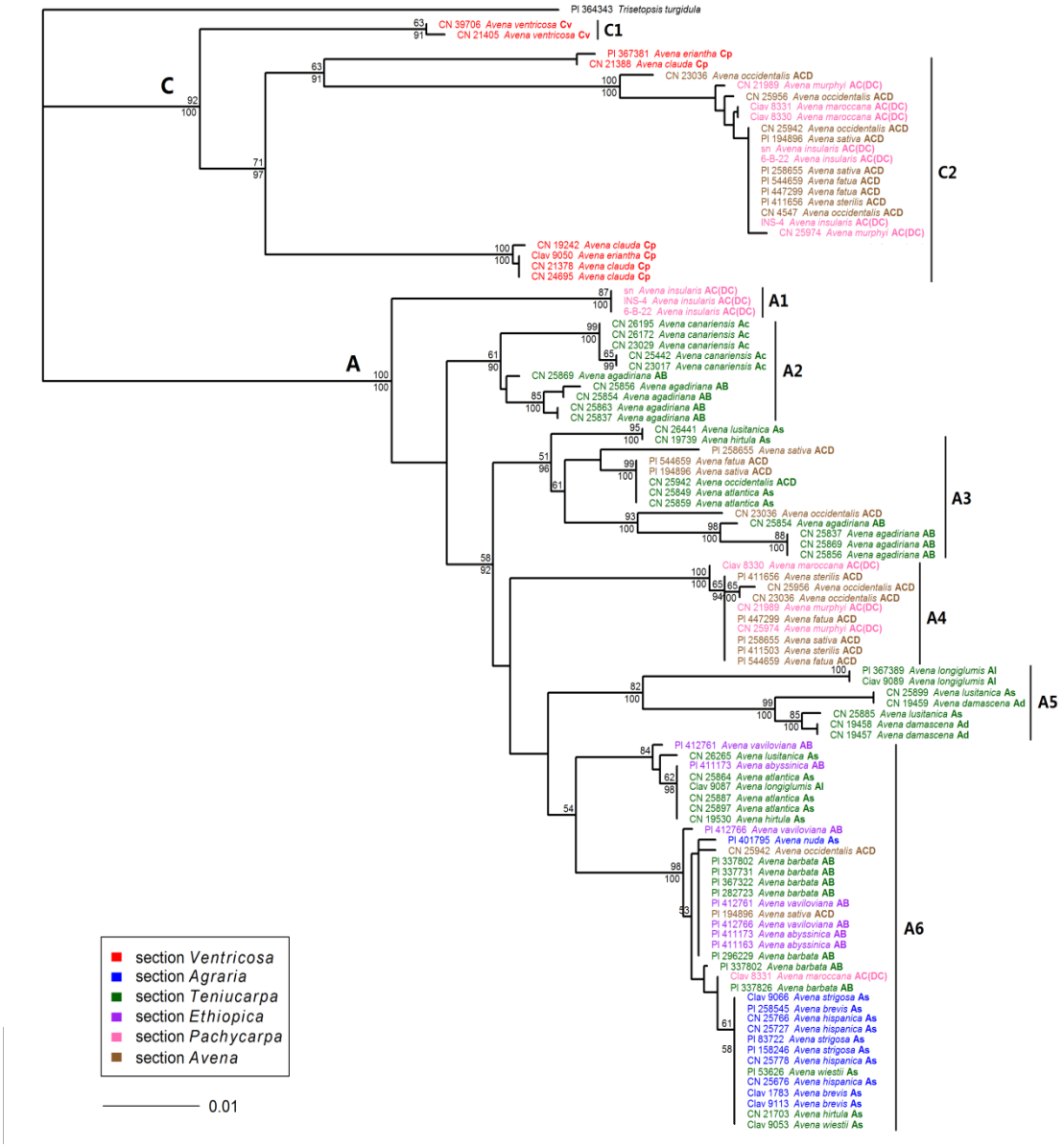


Fig 3. Maximum parsimony tree derived from *Pgk1* sequence data. The tree was constructed using a heuristic search with TBR branch swapping. Numbers above and below the branches are bootstrap support (BS) values ≥50% and Bayesian posterior

probability (PP) values ≥90%. Accession number, species name and haplome are indicated for each taxon.

Fig 3 shows that the *Pgk1* gene sequences from 76 *Avena* accessions were split into two distinct clades with high BS (100% and 92%) and PP (100% and 100%) support. One clade contained all C-genome type sequences, hence referred to as the C genome clade. The other clade contained all sequences from the species carrying the A genome, henceforth, referred to as the A genome clade. The C genome clade was composed of two major subclades. All C_v genome diploids and two C_p genome diploid accessions formed the subclade C1 with 63% BS and 91% PP support, while subclade C2 included four C_p diploids accessions, seven AC(DC) genome tetraploid accessions and nine hexaploid accessions with 71% BS and 97% PP support. The *Pgk1* gene sequences in the A genome clade were further split into six major subclades. The AC(DC) genome tetraploid species *A. insularis* was distinct from the other species, consequently forming a monophyletic clade (A1) with high BS (87%) and PP (100%) support. All five accessions of the A_c genome diploid species *A. canariensis* and one genome homoeologue of the AB genome tetraploid species *A. agadiriana* clustered together into subclade A2. Subclade A3 was composed of four accessions of the AB genome tetraploids *A. agadiriana*, five hexaploid accessions (*A. occidentalis* CN 23036 and CN 25942, *A. sativa* PI 194896 and PI 258655, *A. fatua* PI 544659) and four A_s genome diploid accessions (*A. atlantica* CN25849 and CN 25859, *A. lusitanica* CN 26441, and *A. hirtula* CN 19739). One genome sequence of the AC(DC) genome tetraploids (without *A. insularis*) and the hexaploids formed a

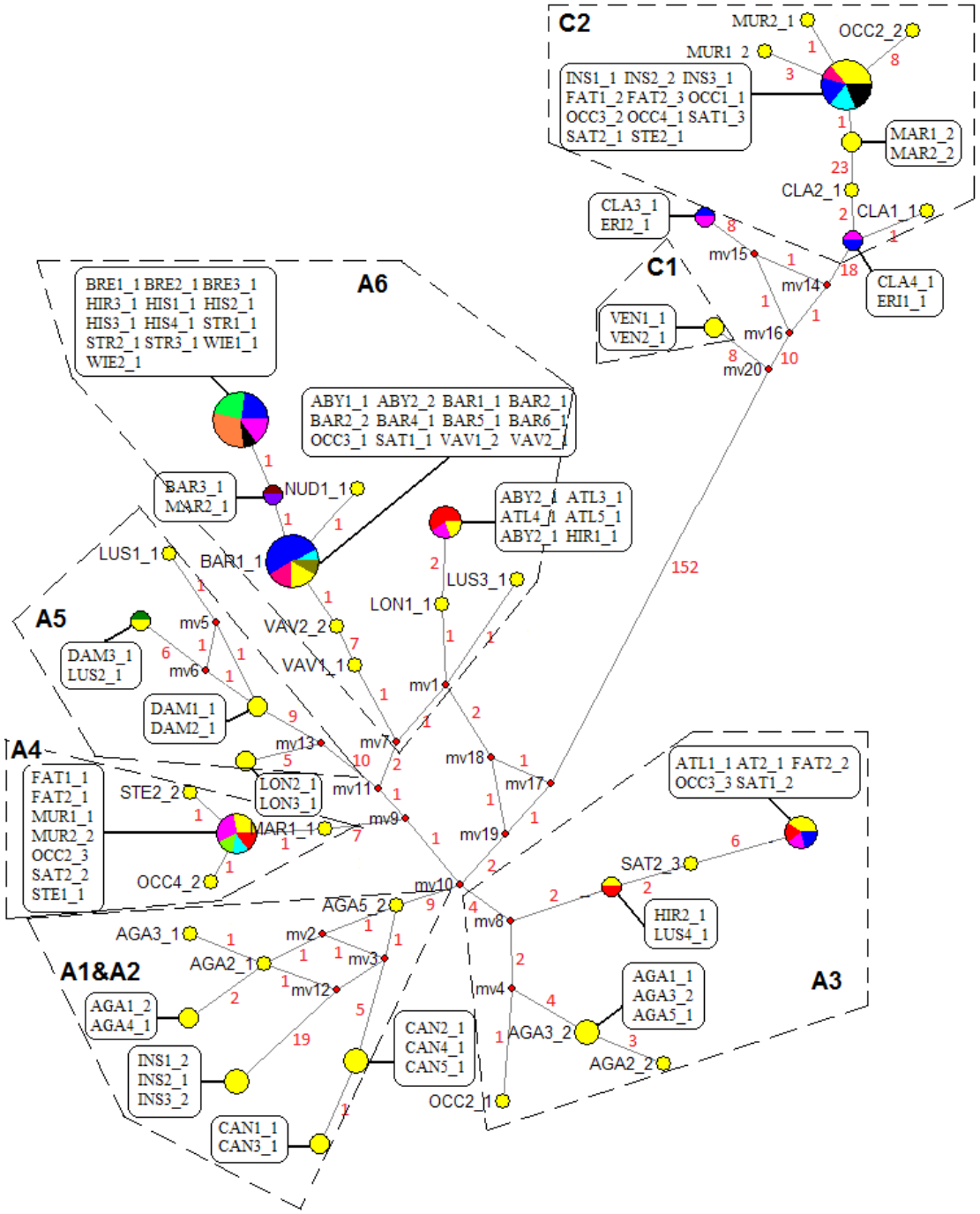
257 homogeneous clade (A4) that was separated from other species with high BS (100%)
258 and PP (100%) support. The subclade A5 consisted of the A_d genome diploid *A.*
259 *damascena*, the A_l genome diploid *A. longiglumis*, and the A_s genome diploid *A.*
260 *lusitanica*. The remaining sequences from the A genome diploids and the AB genome
261 tetraploids (without *A. agadiriana*) formed a relatively broader cluster A6, together
262 with two hexaploid accessions (*A. sativa* PI 194896 and *A. occidentalis* CN 25942)
263 and one AC (DC) genome tetraploid accession (*A. maroccana* CIav 8831).

264 Three groups of haplotypes of *Pgk1* sequences were identified in five
265 hexaploid accessions (*A. fatua* PI 544659, *A. occidentalis* CN 25942, CN 23036, and *A.*
266 *sativa* PI 194896, PI 258655). These sequences fell into four subclades. One group
267 clustered with the C genome diploids in subclade C2, and one group clustered with
268 AC(DC) genome tetraploids in subclade A4. We hypothesize that these two types
269 represent homoeologues from the C and D genomes, respectively. A third and fourth
270 group fell into subclades A3 and A6. Since these two groups are highly separated, it
271 is possible that they represent different A-genome events leading to different
272 hexaploid lineages.

273 Network analysis

274 To gain better insight into relationships within closely related lineages, MJ
275 network reconstruction based on the haplotypes of *Pgk1* sequences was employed.
276 Due to the potential presence of recombination in the exon regions, the intron data
277 was used for MJ network reconstruction. A total of 40 haplotypes were derived from
278 104 *Pgk1* gene sequences (Fig 4). This low level of haplotype diversity demonstrates

279 the high conservation of this gene within genus *Avena*. The MJ network recovered a
280 nearly identical phylogenetic reconstruction to that based on the MP and BI trees,
281 therefore we identified the clades from the MP results (Fig 3) within the MJ network
282 (Fig 4). Based on the topology and frequency of haplotypes, the MJ network was split
283 into two main groups. The two major groups representing two distinct types of
284 haplotypes (A and C genomes) were distinguished due to the 131 bp
285 insertion/deletion. Ten C genome haplotypes were observed, which were much less
286 diverse than the 30 A genome haplotypes. The two main groups were further
287 subdivided into clusters corresponding to the eight MP-based subclades discussed
288 earlier. The only divergence was that the AC(DC) genome tetraploids *A. insularis,*
289 which formed a separate clade (A1) in MP and BI trees, fell into together with the AB
290 genome tetraploid *A. agadiriana* and the A_c genome diploid *A. canariensis* to form a
291 relatively broad cluster in the MJ network (A1&A2).



292 **Fig 4. Median-joining networks based on 40 *Pgk1* gene haplotypes of intron regions**

293 **derived from 26 *Avena* species.** Each circular node represents a single haplotype,

294 with relative size being proportional to the frequency of that haplotype. Distinct

295 colors in the same haplotype node represent different species sharing the same

296 haplotype (colors are arbitrary). Median vectors (mv) represent the putative missing

297 intermediates. Numbers along network branches indicate the number of bases
298 involved in mutations between two nodes. Clusters (surrounded by dashed lines) are
299 named based on clade names shown in the MP tree (Fig 3). Three-letter
300 abbreviations of species names are listed in Table 1. The numbers immediately after
301 each species abbreviation represent different accessions of the same species, and
302 the number following the underscore identifies different haplotypes from the same
303 accession.

304 # Discussion

305 ## Two distinct diploid lineages exist in genus *Avena.*

306 A significant 131 bp insert/deletion separated all *Avena* diploid species into
307 two distinct groups representing the A and C genomes, respectively (Figs 1 and 4).
308 These groups were also separated based on the MP or BI analysis that ignored gaps
309 (Fig 3), indicating that the separation of A and C genomes is the most ancient major
310 articulation in the genus *Avena*, a result that is consistent with most other literature
311 [13, 14, 40]. MJ network analysis revealed that the C genome diploids have much
312 lower levels of haplotype diversity than the A genome diploids. Within the C genome
313 diploids, the C_p genome haplotypes were relatively more diverse than those of the C_v
314 genome. These results might be explained by the geographic distribution of these
315 species. The A genome diploids are distributed in a large region between latitude 20
316 and 40° N, while the C genome diploid species are restricted to a narrow territory
317 along the Mediterranean shoreline [1]. The geographic distributions of the C genome

318 diploid species are overlapping, but the range of the C_p genome diploid species is
319 much broader than that of the C_v genome diploid species [41].

320 The A genome diploid species are the most diverse set of species in genus
321 *Avena*, and chromosome rearrangements have occurred during the divergence of
322 A-genomes from a common progenitor [41], resulting in the subdivision of the A
323 genome into five types, of which we have investigated four. Our results showed that
324 species with genome types A_c, A_l, and A_d formed groups that correspond well with
325 previously reported structural differences. However, the A_s genome diploids appear
326 to be much more diverse than previously reported, and are scattered into different
327 subclades (Fig 3). Baum [1] divided all A_s genome diploids into two sections, section
328 *Agraria* and section*Tenuicarpa.* All species of section *Agraria* have florets with a
329 domesticated (non-shattering) base, whereas the other A_s species share relatively
330 narrow spikelets. However, classification based on simple morphological traits is
331 increasingly controversial. In this study, the A_s genome diploid species of section
332 *Agraria* showed high degree of genetic homogeneity, consistently forming their own
333 subclade A6, but other A_s genome species in section *Tenuicarpa* did not have their
334 own subclade. *A. wiestii* showed a close relationship with the species of section
335 *Agraria*, suggesting that it may be better-classified within that section. This result is
336 in agreement with previous studies based on RAPD (Perchuk et al. 2002) and
337 karyotypic comparisons (Badaeva et al. 2005). Accessions of the other two A_s
338 genome species of section *Tenuicarpa* (*A. atlantica* and *A. hirtula*) were scattered
339 into different subclades. These results were also observed in other studies (Peng et

al. 2010, Yan et al. 2014). *A. lusitanica*, another A_s species of section *Tenuicarpa,* was diverged from other A_s species, but showed a close relationship to those with the A_d genome species *A. damascena*. This divergence has also been observed in many other studies [8, 9, 14, 40]. These, and other incongruences between morphological characters and genetic differences raise questions about appropriate taxonomical classifications among A_s genome species.

The A_s and A_c genomes played roles in the AB tetraploid formation.

Four recognized species have been proposed to have an AB genome composition. Of these, *A. barbata*, *A. abyssinica* and *A. vaviloviana* are grouped into a biological species known as the *barbata* group, while *A. agadiriana* is distinct [25, 42]. Our results confirmed the reported structural differences between these two groups (Fig 3). Two different *Pgk1* gene sequences were detected from most of the AB genome tetraploids, supporting their allotetraploid origins. However, the genomes of *A.barbata* showed the least divergence, with only one of six *A. barbata* accessions providing multiple sequences, both of which were very similar. It seems that little divergence has occurred within the genome of *A. barbata* compared with that of *A. abyssinica* and *A.vaviloviana*, suggesting that *A. barbata* is the ancestral version of the species within the *barbata* group. This is supported by two lines of evidence. First, both *A. abyssinica* and *A.vaviloviana* are semi-domesticated forms that occur almost exclusively in Ethiopia, whereas the wild *A. barbata* are more geographically distributed, but can still be found close to the *abyssinica* and

362 *vaviloviana* forms [43]. The second line of evidence was provided by FISH and
363 Southern hybridization [17], which found some B chromosomes of *A. vaviloviana* are
364 involved in inter-genomic translocations, while these rearrangements were not
365 detected in *A. barbata*. There is little doubt that the A genome diploids have been
366 involved in the formation of the *barbata* species. Some studies have suggested that
367 both the A and B genomes of *barbata* species are diverged A_s genomes [16, 23, 44],
368 while some others proposed that the B genome might have originated from other A
369 genome diploid species [24, 25, 45]. In this study, both types of *Pgk1* sequences
370 detected from the *barbata* group showed high degree of genetic homogeneity with
371 the A_s genome diploids (Fig 3), thus it was impossible to determine which type
372 represents the A or B genome.
373 The recently discovered tetraploid species *A. agadiriana* was also proposed
374 to have an AB genome composition because of its high affinity with *A. barbata* [23].
375 However, this designation has been questioned due to chromosomal divergences
376 between *A. agadiriana* and the *barbata* species, as revealed by cytological studies
377 [45, 46] and by molecular data [9, 13, 14]. In the current study, two distinct types of
378 *Pgk1* sequences were obtained in *A. agadiriana*. One copy clustered with the A_c
379 genome species *A. canariensis*, whereas the other copy fell into cluster A3 with the
380 A_s species *A. atlantica*, *A. hirtula*, *A. lusitanica*, and the hexaploids *A. occidentalis*, *A.*
381 *fatua* and *A. sativa* (Fig 3). These results were in agreement with our previous
382 studies based on nuclear *Acc1* gene [14] and GBS markers [9], and they support the
383 proposal that *A. agadiriana* contains a different combination of A and/or B genomes

384 from the *barbata* group, and that one of its two genomes originates from the A_c
385 genome species *A. canariensis*, whereas the other one is closely related to the A_s
386 species.

387 The tetraploid species *A. maroccana* and *A. murphyi* are
388 closely related to the hexaploids, while *A. insularis* is
389 diverged.

390 The other tetraploid group (*Avena* sect. *Pachycarpa*) contains three species, *A.*
391 *maroccana*, *A. murphyi,* and the recently discovered *A. insularis*. Initial studies based
392 on genomic in situ hybridization [47] supported an AC genome designation for these
393 species. However, this designation has been challenged by FISH analysis, which has
394 revealed that this set of tetraploid species, like the D chromosomes of the hexaploid
395 oats, lacks a repetitive element that is diagnostic of the A genome [18]. This,
396 together with other molecular evidence [14, 48] and our recent whole-genome
397 analysis based on GBS markers [9], suggests that these tetraploid species contain the
398 genome designated as D in hexaploid oats, and that they are more properly
399 designated as DC genome species.

400 In the present study, two distinct *Pgk1* homoeologues were detected in each
401 of the three AC(DC) species, with each pair falling consistently into two clusters
402 within the C and the A genome clades, respectively (Fig 3). The C-copy sequences of
403 these tetraploids clustered consistently with the C-type homoeologues of the
404 hexaploids, while the A/D genome homoeologues, with the exception of these from
405 *A. insularis* and one sequence from *A. maroccana* (CIav 8331) fell into subclade A4

406 along with a set of sequences from the hexaploid oats (Fig 3). Considering that the
407 other *Pgk1* gene sequences from the hexaploid oats clustered with the C or A
408 genome diploids, we deduced that the sequences falling in subclade A4 must
409 represent the D genome homoeologues of the hexaploids and of the AC(DC) species
410 *A. maroccana* and *A. murphyi*. This result is not fully consistent with our previous
411 GBS study: although *A. maroccana* and *A. murphyi* were very similar to hexaploid oat
412 and were designated as DC genomes, our GBS work suggested that *A. insularis* was
413 also a DC genome that was even more similar to the hexaploids [9]. Examining the
414 existing literature, all three of these tetraploid species have variously been
415 considered as the tetraploid ancestor of the hexaploids [4, 9, 49]. In view of the
416 genome structure of these tetraploids [24, 50] and the meiotic chromosome paring
417 of their interspecific hybrids [51], all of these tetraploids are proposed to have
418 diverged from a common ancestral tetraploid after the occurrence of some large
419 chromosome rearrangements [24, 50]. However, it cannot be ruled out that these
420 tetraploids might have originated independently from different diploid ancestors,
421 since they have shown close relationships with different diploid species [40].
422 Interestingly, in network analysis (Fig 4), the A/D-type homoeologues of *A. insularis*
423 fell into a group with the A_c genome species *A. canariensis* and the AB genome
424 species *A. agadiriana*. In fact, previous studies have revealed that *A. canariensis* is
425 closely related to the DC genome tetraploids [15]. These results suggest a possibility
426 that *A. canariensis* was involved in contributing an early version of a D genome in all
427 three AC(DC) genome tetraploids. Nevertheless, we do not have an explanation for

why the D genome copy of *Pgk1* in *A. insularis* could have diverged so far from the version found in the hexaploids, especially since the C genome copies remain identical.

The genome origins of the hexaploid species.

It is now generally accepted that two distinct steps were involved in the evolution of hexaploid oats. The first step would have been the formation of a DC genome hybrid from ancestral D and C genome diploids, followed by doubling of the chromosomes to form an allotetraploid. The second step would have involved hybridization of a DC tetraploid with a more recent A genome diploid, followed by doubling of the triploid hybrid [9, 13].

The diploid progenitor of the hexaploid C genome was probably restricted to the narrow geographic range where the three extant C genome diploids are distributed. However, numerous inter-genomic translocations among hexaploid chromosomes [9, 11, 52, 53]have deceased the homology between the C genome diploids and the hexaploid C genome, making the identification of the C genome donor of the hexaploids challenging. In this study, the C_p genome species shared the highest degree of genetic similarity with both the DC genome tetraploids, as well as with the hexaploids, leading us to conclude that a C_p genome species was the C genome donor of the polyploids. This conclusion is supported by other evidence from nuclear genes [13, 54]. This is important, since it was recently demonstrated that the maternal tetraploid and hexaploid genomes originated from an A genome species, not from a C genome species [55], rendering comparisons to the C_v *vs* C_p

450 maternal genomes irrelevant in determining the origin of the nuclear C genome in
451 the hexaploids.

452 The A genome origin of the hexaploids remains a matter of debate, and many
453 A genome diploids have been suggested as putative diploid progenitors, as
454 summarized by Peng et al [13]. FISH analysis showed that an A_s-specific DNA repeat
455 was restricted to the A_s and A_l genomes, as well as the hexaploid A genome [18]. In
456 this study, a close relationship between the A_s genome diploid *A. atlantica* was
457 observed for some hexaploid haplotypes in the phylogenetic tree (Fig 3) and the MJ
458 network (Fig 4). An *A. atlantica* genome origin is consistent with previous studies
459 based on IGS-RFLP analysis [12] and the *ppcB1* gene [40]. However, there is evidence
460 in our work that some hexaploids may have an alternate A genome origin, closer to
461 the *Agraria* section of A_s diploids. The presence of multiple A genome origins could
462 explain variable results that have been reported in studies of hexaploid phylogeny.

463 In this study, strong evidence is presented for a D genome origin in the
464 tetraploids *A. maroccana* and *A. murphyi* (Figs 3-4). However, these D genome
465 sequences did not show a close relationship with any diploid species investigated in
466 this study. Other than the discrepancy with *A. insularis*, this result is consistent with
467 our recent GBS study [9]. One factor that may hinder the discovery of a D genome
468 progenitor is the presence of inter-genomic translations among all three genomes in
469 the hexaploid [9, 53]. Two hexaploid accessions (*A.occidentalis* CN 25942 and *A.*
470 *sativa* PI 194896) did not contribute haplotypes that clustered with the putative D
471 genome sequences (Subclade A4 in Fig 3). Although this may be a result of

472 incomplete sampling, it may also result from inter-genomic translations that have
473 duplicated or eliminated copies of *Pgk1.*

474 In conclusion, this is the most comprehensive study to date that investigates
475 a phylogeny in genus *Avena* using a single informative nuclear gene. It confirms or
476 clarifies most previous work, and presents strong evidence in support of a working
477 hypothesis for the origin of hexaploid oat. However, many questions still remain, and
478 these will be best addressed through further studies involving multiple nuclear genes
479 or whole genomes. We are collaborating on work that will provide exome-based
480 gene diversity studies, but this work will require complete hexaploid reference
481 sequences before it can be properly analyzed. Such reference sequences are
482 currently in progress, so the next few years may see a revolution in our
483 understanding of *Avena* phylogeny. Nevertheless, as many researcher in this field are
484 aware, the polyploid species in this genus have experienced extensive chromosome
485 rearrangement, which will continue to complicate phylogenetic studies. It may even
486 be necessary to generate a pan-genome hexaploid reference sequence before
487 definitive statements can be made.

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