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# Animal, fungi, and plant genome sequences harbour different non-canonical splice sites

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# Abstract

Most protein encoding genes in eukaryotes contain introns which are interwoven with exons. After transcription, introns need to be removed in order to generate the final mRNA which can be translated into an amino acid sequence by the ribosome. Precise excision of introns by the spliceosome requires conserved dinucleotides which mark the splice sites. However, there are variations of the highly conserved combination of GT at the 5' end and AG at the 3' end of an intron in the genome. GC-AG and AT-AC are two major non-canonical splice site combinations which are known for many years. During the last few years, various minor non-canonical splice site combinations were detected with all possible dinucleotide permutations. Here we expand systematic investigations of non-canonical splice site combinations in plant genomes to all eukaryotes by analysing fungal and animal genome sequences. Comparisons of splice site combinations between these three kingdoms revealed several differences such as a substantially increased CT-AC frequency in fungal genomes. In addition, high numbers of GA-AG splice site combinations were observed in two animal species. In depth investigation of splice site usage based on RNA-Seq read mappings indicates a generally higher flexibility of the 3' splice site compared to the 5' splice site.

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# Introduction

Splicing, the removal of introns after transcription, is an essential step during the generation of mature mRNAs in eukaryotes. This process allows variation which provides the basis for quick adaptation to changing conditions [1, 2]. Alternative splicing, e.g. skipping exons, results in an enormous diversity of synthesized proteins and therefore substantially expands the diversity of products encoded in eukaryotic genomes [3–6]. The full range of functions as well as the evolutionary relevance of introns are still under discussion [7]. However, introns are energetically expensive for the cell to maintain as the transcription of introns costs time and energy and the removal of introns has to be exactly regulated [8]. Dinucleotides at both intron/exon borders mark the splice sites and are therefore highly conserved [9]. GT at the 5' end and AG at the 3' end of an intron form the canonical splice site combination on DNA level. More complexity arises through non-canonical splice site combinations, which deviate from the highly conserved canonical one. Besides the major non-canonical splice site combinations GC-AG and AT-AC, several minor non-canonical splice site combinations have been detected before [9, 10].

Furthermore, the position of introns in homologous genes across organisms, which diverged 500-1500 million years ago, are not conserved [11]. In addition, many intron sequences mutate at a higher rate due to having much less of an impact on an organism's reproductive fitness compared to a mutation located within an exon [12]. These factors, along with the existence of several non-cannonical splice sites, make the complete prediction of introns, even in non-complex organisms like yeast, almost impossible [13, 14]. Moreover, most introns which can be predicted computationally still lack experimental support [15].

Splice sites are recognised during the splicing process by a complex of snRNAs and proteins, the spliceosome [16]. U2-spliceosome and U12-spliceosome are two subtypes of this complex which comprise slightly dif-

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ferent proteins with equivalent functions [17–19]. Although the terminal dinucleotides are important for the splicing process, these splice sites are not sufficient to determine which spliceosome is processing the enclosed intron [20]. This demonstrates the complexity of the splicing process which involves additional signals present in the DNA. Even though multiple mechanisms could explain the splicing process, the exact mechanism of non-canonical splicing is still not completely resolved [5].

Branching reaction and exon ligation are the two major steps of splicing [21, 22]. In the branching reaction, the 2'-hydroxyl group of the branch-point adenosine initiates an attack on the 5'-phosphate of the donor splice site [23, 24]. This process leads to the formation of a lariat structure. Next, the exons are ligated and the intron is released through activity of the 3'-hydroxyl group of the 5'exon at the acceptor splice site [21].

Previous in-depth analyses of non-canonical splice sites in fungi and animals were often focused on a single or a small number of species [9, 25, 26]. Several studies focused on canonical GT-AG splice sites but neglected non-canonical splice sites [27, 28]. Our understanding of splice site combinations is more developed in plants compared to other kingdoms [10, 29–33]. Previous works reported 98 % GT-AG splice site combinations in fungi [25], 98.7 % in plants [10] and 98.71 % in animals [9]. Consequently, the proportion of non-canonical splice sites is around or below 2 % [9, 10, 25]. To the best of our knowledge, it is not known if the value reported for mammals is representative for all animals. The combined proportion of minor non-canonical splice sites is even lower e.g. 0.09 % in plants, but still exceeding the frequency of the major non-canonical AT-AC splice sites [10]. Despite this apparently low frequency, non-canonical splice site combinations have a substantial impact on gene products, especially on exon-rich genes [10]. About 40 % of genes with 40 exons are affected (AdditionalFile 11).

Consideration of non-canonical splice sites is important for gene predic-

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tion approaches, because these sites cannot be identified *ab initio* [29]. Moreover, as many human pathogenic mutations occur at the donor splice site [34], it is of great interest to understand the occurence and usage of non-canonical splice sites. Therefore, several non-canonical splice sites containing AG at the acceptor site were investigated in human fibroblasts [34]. Alongside this, fungi are interesting due to pathogenic properties and importance in the food industry [35]. Since splicing leads to high protein diversity [3–6], the analysis of splicing in fungi is important with respect to biotechnological applications e.g. development of new products.

In this study, a collection of annotated genome sequences from 130 fungi and 489 animal species was screened for canonical and non-canonical splice site combinations. RNA-Seq data sets were harnessed to identify biologically relevant and actually used splice sites. Non-canonical splice site combinations, which appeared at substantially higher frequency in a certain kingdom or species, were analysed in detail. As knowledge about splice sites in plants was available from previous investigations [10, 29], a comparison between splice sites in fungi, animals and plants was performed.

# Results and Discussion

## Analysis of non-canonical splice sites

In total, 64,756,412 and 2,302,340 splice site combinations in animals and fungi, respectively, were investigated based on annotated genome sequences (AdditionalFile 1 and 2). The average frequency of the canonical splice site combination GT-AG is 98.3 % in animals and 98.7 % in fungi, respectively. These values exceed the 97.9 % previously reported for plants [10], thus indicating a generally higher frequency of non-canonical splice site combinations in plants. As previously speculated [10], a gen-

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erally more complex splicing system in plants could be an adaptation to changing environments. Since most plants are not able to change their geographic location, the tolerance for unfavourable conditions should be stronger than in animals. The lower proportion of non-canonical splice sites in fungi compared to animals seems to contradict this hypothesis. However, the genome size and complexity needs to be taken into account here. The average animal genome is significantly larger than the average fungal genome (Mann-Whitney U-Test; p=5.64e-68) (AdditionalFile 3). Average percentages of the most important splice site combinations were summarized per kingdom and over all analysed genomes (Table 1). The number of canonical and non-canonical splice site combinations per species was also summarized (AdditionalFile 4 and 5). A higher percentage of non-canonical splice sites was observed in animals in comparison to fungi. Several species strongly exceeded the average values for major and minor non-canonical splice sites. The fungal species *Meyerozyma guilliermondi* shows approximately 6.67 % major and 13.33 % minor non-canonical splice sites. *Eurytemora affinis* and *Oikopleura dioica* reveal approximately 10 % minor non-canonical splice sites. In summary, the observed frequencies of canonical and major non-canonical splice site combinations are similar to the pattern previously reported for plants [10], but some essential differences and exceptions were found in animals and fungi.

**Table 1: Splice site combination frequencies in animals, fungi, and plants.** Only the most frequent combinations are displayed here and all minor non-canonical splice site combinations are summarized as one group ("others"). A full list of all splice site combinations is available (AdditionalFile 6 and 7).

| | GT-AG | GC-AG | AT-AC | others |
|---|---|---|---|---|
| **animals** | 98.334 % | 0.983 % | 0.106 % | 0.577 % |
| **fungi** | 98.715 % | 1.009 % | 0.019 % | 0.257 % |
| **plants** | 97.886 % | 1.488 % | 0.092 % | 0.534 % |
| **all** | 98.265 % | 1.074 % | 0.101 % | 0.560 % |

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Different properties of the genomes of all investigated species were analysed to identify potential explanations for the splice site differences (AdditionalFile 8 and 9). In fungi, the average number of introns per gene is 1.49 and the average GC content is 47.1 % ($\pm$7.39). In animals, each gene contains on average 6.95 introns and the average GC content is 39.4 % ($\pm$3.87). This difference in the GC content could be associated with the much lower frequency of AT-AC splice site combinations and the higher frequency of CT-AC splice site combinations in fungi (Figure 1). CT-AC has a higher GC content than the AT rich AT-AC splice site combination. A generally higher GC content could result in the higher GC content within splice site combinations due to the overall mutations rates in these species.

A comparison of the genome-wide GC content to the GC content of all splice sites revealed a weak correlation in the analysed fungi ($r \approx 0.236$, $p \approx 0.008$). Species with a high genomic GC content tend to show a high GC content in the splice site combinations in the respective species. A similar correlation ($r \approx 0.4$, $p < 0.001$) was found in plant and animal species as well (AdditionalFile 10). Additionally, the GC content in fungal genomes is substantially exceeding the average GC content of plant and animal genomes.

The most frequent non-canonical splice site combinations show differences between animals, fungi, and plants (Figure 1). In fungal species, the splice site CT-AC is more frequent than the splice site combination AT-AC. Regarding the splice site combination GA-AG in animals, two outliers are clearly visible: *Eurytemora affinis* and *Oikopleura dioica* show more GA-AG splice site combinations than GC-AG splice site combinations.

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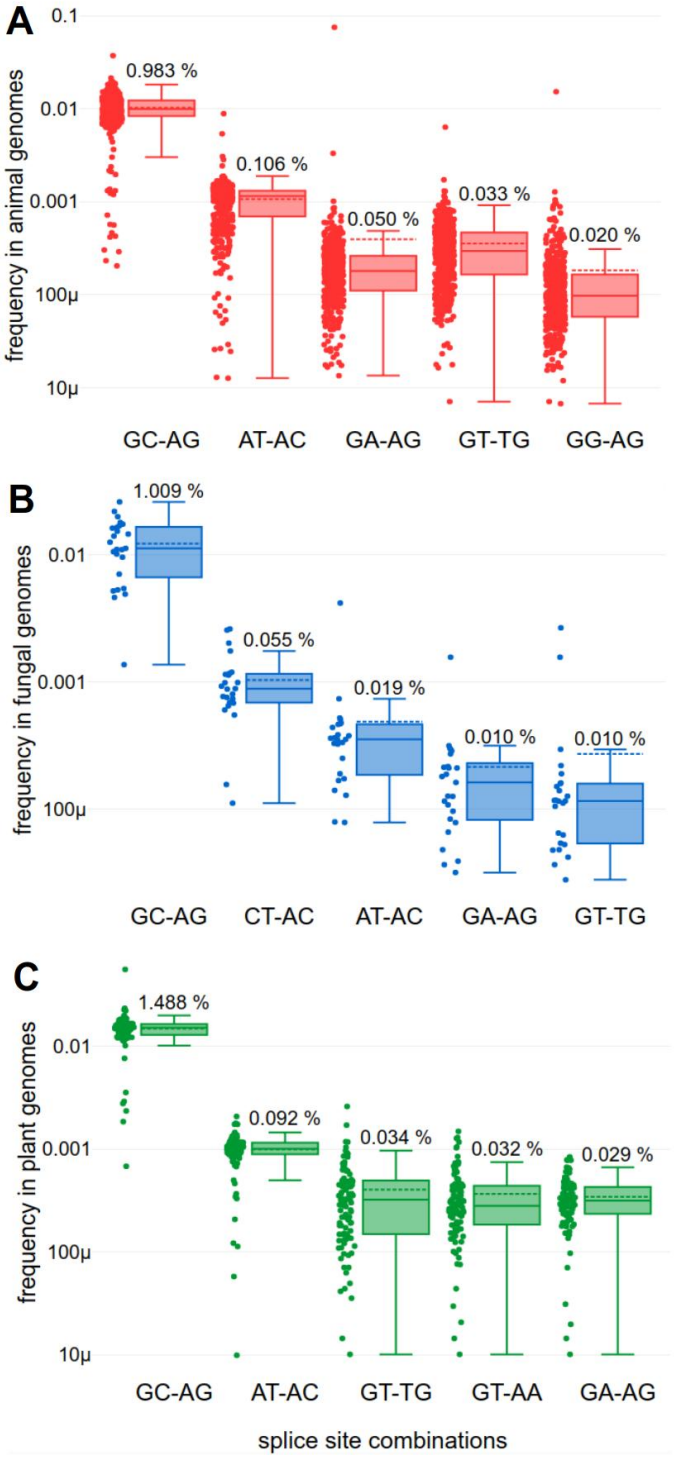


**Figure 1: Frequencies of non-canonical splice site combinations in animals, fungi, and plants.** The frequency of non-canonical splice site combinations across the 489 animal (red), 130 fungal (blue) and 121 plant (green) genomes is shown. Normalization of the absolute number of each splice site combination was performed per species based on the total number of splice sites. The frequency of the respective splice site combination of each species is shown on the left hand side and the percentage of the respective splice site combination on top of each box plot.

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Despite overall similarity in the pattern of non-canonical splice site combinations between kingdoms, specific minor non-canonical splice sites were identified at much higher frequency in some fungal and animal species. First, RNA-Seq data was harnessed to validate these unexpected splice site combinations. Next, the frequencies of selected splice site combinations across all species of the respective kingdom were calculated. The correlation between the size of the incorporated RNA-Seq data sets and the number of supported splice sites was examined as well (AdditionalFile 11). In animals, there is a correlation ($r \approx 0.417$, $p \approx 0.022$) between number of supported splice sites and total number of sequenced nucleotides in RNA-Seq data. For fungi, no correlation between number of splice sites and size of the RNA-Seq data sets could be observed. It is important to note that the the number of available RNA-Seq data sets from fungi was substantially lower. Further, analysis of introns with canonical and non-canonical splice site combinations, respectively, revealed that a higher number of introns is associated with a higher proportion of non-canonical splice sites (AdditionalFile 12).

## High diversity of non-canonical splice sites in animals

Kupfer *et al.* suggested that splicing may differ between fungi and vertebrates [25]. Our results indicate substantial differences in the diversity of splice site combinations other than GT-AG and GC-AG in fungi ($H' \approx 0.0277$) and animals ($H' \approx 0.0637$) (Kruskal-Wallis: $p \approx 0.00000$). Besides the overall high proportion of minor non-canonical splice sites (Table 1), differences between species are high (Figure 1). The slightly higher interquartile range of splice site combination frequencies in animal species and especially in plant species (Figure 1A and C), together with the relatively high frequency of ”other” splice sites in animals and plants (Table 1) suggest more variation of splice sites in the kingdoms of animals and plants compared to the investigated fungal species. Thus, the high diversity of splice sites could be associated with the higher complexity of animal and plant genomes. In addition, the difference in prevalence be-

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tween the major non-canonical splice site combination GC-AG and minor non-canonical splice site combinations is smaller in animals compared to fungi and plants (Figure 1).

GA-AG is a frequent non-canonical splice site combination in some animal species. Two species, namely *Eurytemora affinis* and *Oikopleura dioica*, showed a much higher abundance of GA-AG splice site combinations compared to the other investigated species (Figure 1A). RNA-Seq reads support 5,795 (28.68 %) of all GA-AG splice site combinations of these species. In both species, the number of the GA-AG splice site combination exceeds the number of the major non-canonical splice site combination GC-AG.

For *Eurytemora affinis*, the high frequency of the GA-AG splice site combinations was described previously for 36 introns [36]. We quantified the proportion of GA-AG splice site combinations to 3.2 % (5,345) of all 166,392 supported splice site combinations in this species. The donor splice site GA is flanked by highly conserved upstream AG and a downstream A (Figure 2).

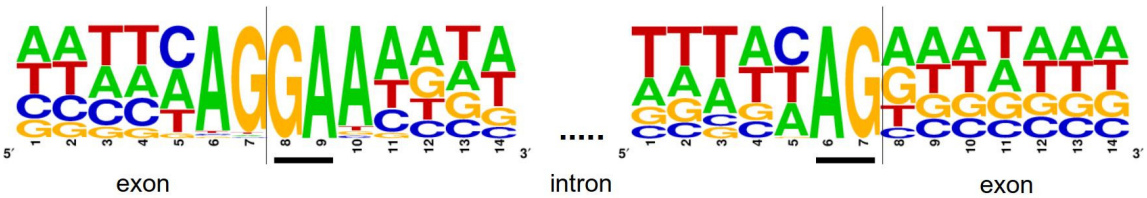


**Figure 2: Flanking positions of GA-AG splice site combinations in *Eurytemora affinis* and *Oikopleura dioica*.** All 5,795 supported splice site combinations of these two species were investigated. Seven exonic and seven intronic positions are displayed at the donor and acceptor splice sites. Underlined bases represent the terminal dinucleotides of the intron i.e. the donor and acceptor splice site.

Efficient splicing of the splice site combination GA-AG was detected in human fibroblast growth factor receptor genes [37]. Further, it was suggested that this splicing event is, among other sequence properties, dependent on a canonical splice site six nucleotides upstream [37], which does not exist

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in the species investigated here (Figure 2). An analysis of all five potential U1 snRNAs in this species did reveal one single nucleotide polymorphism in the binding site of the 5' splice site from C to T in one of these U1 snRNAs. This could result in the binding of AG/GGAAGT or AGG/GAAGT instead of AG/GTAAGT. Although this would imply an elegant way for the splicing of GA-AG splice sites, the same variation was also detected in putative human U1 snRNAs. Therefore, another mechanism seems to be responsible for splicing of introns containing the GA-AG splice site combination.

## CT-AC is a frequent splice site combination in fungi

Although the general frequency pattern of fungal splice site combinations is similar to plants and animals, several fungal species displayed a high frequency of minor non-canonical CT-AC splice site combinations. This co-occurres with a lower frequency of AT-AC splice site combinations. Non-canonical splice sites in fungi were, so far, only described in studies which focussed on a single or a few species. An analysis in the oomycota species *Phytophthora sojae*, which is a fungus-like microorganism [38, 39], revealed 3.4 % non-canonical splice site combinations GC-AG and CT-AC [40]. Our findings indicate, that the minor non-canonical splice site combination CT-AC occurs with a significantly (Mann-Whitney U-Test; $p \approx 0.00035$) higher frequency than the major non-canonical splice site combination AT-AC. In contrast, the frequency of AT-AC in animals and plants exceeds the CT-AC frequency significantly ($p < 0.001$) (Figure 3A). For the splice site combination CT-AC a sequence logo, which shows the conservation of this splice site in four selected species, was designed (Figure 3B). In summary, we conclude that CT-AC is a major non-canonical splice site combination in fungi, while AT-AC is not.

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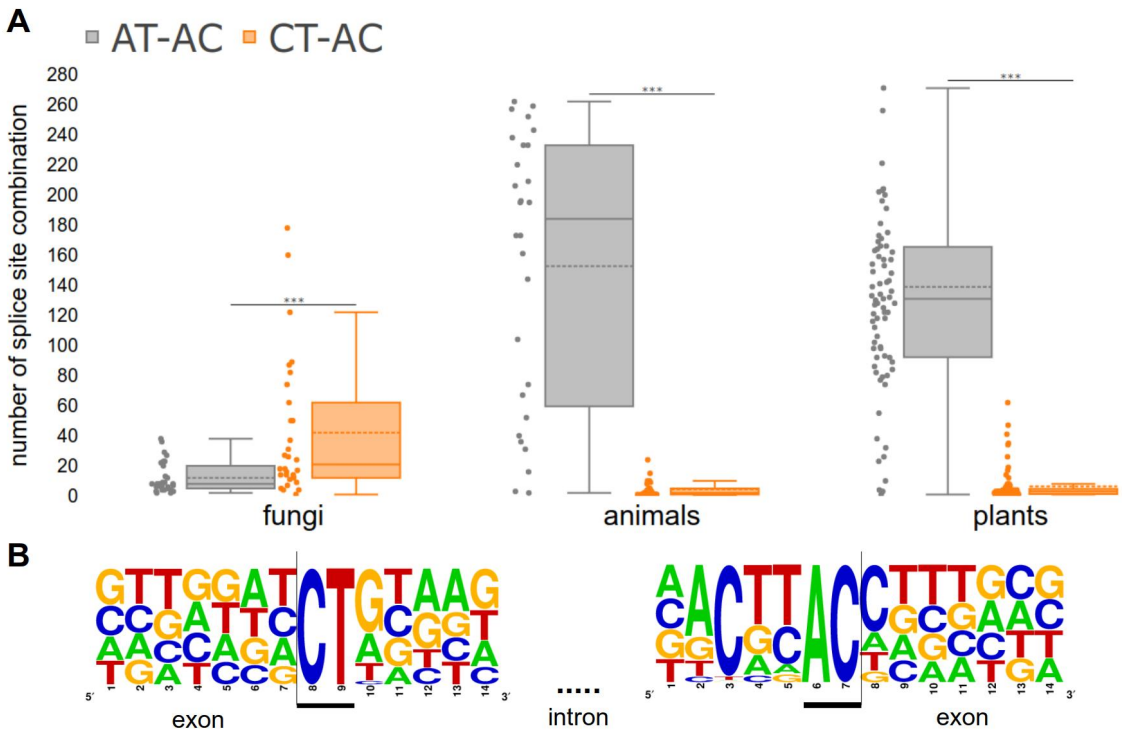


**Figure 3: CT-AC frequency exceeds AT-AC frequency in fungi.** A) Number of the minor non-canonical splice site combination CT-AC in comparison to the major non-canonical splice site combination AT-AC in each kingdom ($p<0.001$). B) Sequence logo for the splice site combination CT-AC in four selected fungal species (*Alternaria alternata*, *Aspergillus brasiliensis*, *Fomitopsis pinicola* and *Zymoseptoria tritici*). In total, 67 supported splice sites with this combination were used to generate the sequence logo.

The highest frequencies of the splice site combination CT-AC, supported by RNA-Seq reads, were observed in *Alternaria alternata*, *Aspergillus brasiliensis*, *Fomitopsis pinicola* and *Zymoseptoria tritici* (approx. 0.08 - 0.09 %). As AT-AC was described as major non-canonical splice site, these findings indicate a different splice site pattern in fungi compared to animals and plants (Figure 3).

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## Intron size analysis

In total, 8,060,924, 737,783 and 2,785,484 transcripts across animals, fungi and plants, respectively, were selected to check whether the intron lengths are multiples of three. Introns with this property could be kept in the final transcript without causing a shift in the reading frame. There is no significant difference between introns with different splice site combinations (Table 2). The ratio of introns with a length divisible by 3 is very close to 33.3 % which would be expected based on an equal distribution. The only exception are minor non-canonical splice site combinations in fungi which are slightly less likely to occur in introns with a length divisible by 3.

**Table 2: Proportion of introns with length divisible by 3. The results of intron length analysis for selected splice site combinations for animals, fungi and plants are shown.**

| | splice site combination | frequency of introns divisible by 3 | total number of introns divisible by 3 |
|---|---|---|---|
| **animals** | **GT-AG** | 0.333862150381 | n=63677347 |
| | **AT-AC** | 0.325106284189 | n=68919 |
| | **GC-AG** | 0.330352389911 | n=636823 |
| | **others** | 0.327633755094 | n=496411 |
| **fungi** | **GT-AG** | 0.33932356858 | n=2273756 |
| | **AT-AC** | 0.331775700935 | n=428 |
| | **GC-AG** | 0.333577333793 | n=23224 |
| | **others** | 0.3125 | n=6240 |
| **plants** | **GT-AG** | 0.332967299596 | n=14227286 |
| | **AT-AC** | 0.326150175229 | n=13411 |
| | **GC-AG** | 0.329271562364 | n=216326 |
| | **others** | 0.323971037399 | n=93638 |

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## Conservation of non-canonical splice site combinations across species

In total, *A. thaliana* transcripts containing 1,073 GC-AG, 64 AT-AC and 19 minor non-canonical splice sites were aligned to transcripts of all plant species. Homologous intron positions were checked for non-canonical splice sites. GC-AG splice site combinations were conserved in 9,830 sequences, matched with other non-canonical splice site combinations in 121 cases, and aligned to GT-AG in 13,045 sequences. Given that the dominance of GT-AG splice sites was around 98 %, the number observed here indicates a strong conservation of GC-AG splice site combinations. AT-AC splice site combinations were conserved in 967 other sequences, matched with other non-canonical splice site combinations in 93 cases, and aligned to GT-AG in 157 sequences. These numbers indicate a conservation of AT-AC splice site combinations, which exceeds the conservation of GC-AG splice site combinations substantially. Minor non-canonical splice sites were conserved in 48 other sequences, matched with other non-canonical splice site combinations in 64 cases, and were aligned to a canonical GT-AG splice site in 213 cases. This pattern suggests that most non-canonical splice site combinations are either (A) mutations of the canonical ones or (B) mutated towards GT-AG splice site combinations.

The power of this analysis is currently limited by the quality of the alignment. Although splice site combinations should be aligned properly in most cases, small differences in the number could be caused by ambiguous situations. It is likely that both hypothesis stated above are partly valid. To assign each splice site combination to A or B, a manual inspection of the observed phylogenetic pattern would be required.

## Usage of non-canonical splice sites

Non-canonical splice site combinations were described to have regulatory roles by slowing down the splicing process [41]. Previous reports

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also indicated that non-canonical splice site combinations might appear in pseudogenes [9, 10]. To analyse a possible correlation of non-canonical splice sites with low transcriptional activity, we compared the transcript abundance of genes with non-canonical splice site combinations to genes with only canonical GT-AG splice site combinations (Figure 4A). Genes with at least one non-canonical splice site combination are generally less likely to be lowly expressed than genes with only canonical splice sites. While this trend holds true for all analysed non-canonical splice site combination groups, GC-AG and AT-AC containing genes display especially low proportions of genes with low FPKMs. We speculate that a stronger transcriptional activity of genes with non-canonical splice sites compensates for lower turnover rates in the splicing process. The regulation of the genes might be shifted from the transcriptional to the post-transcriptional level. This trend is similar for animals and plants (AdditionalFile 13). In fungi, genes with minor non-canonical splice sites display relatively high proportions of genes with low FPKMs.

Moreover, a higher number of non-canonical splice sites per gene is associated with a lower expression. This leads to the suggestion, that non-canonical splice sites occur more often within pseudogenes.

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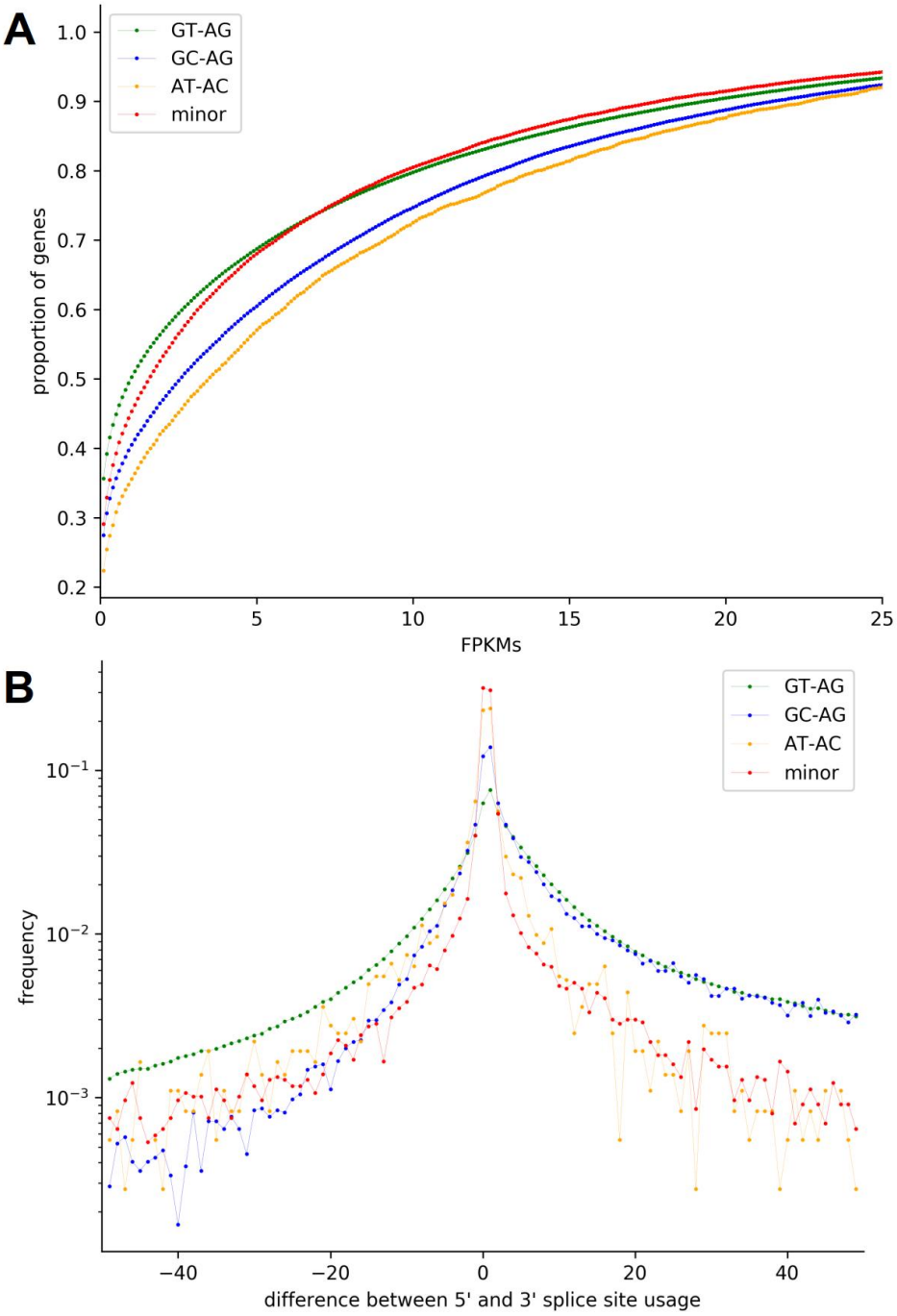


**Figure 4: Usage of non-canonical splice sites in plant species.** A) Comparison of the transcript abundance (FPKMs) of genes with non-canonical splice site combinations to genes with only canonical GT-AG splice site combinations. GC-AG and AT-AC containing genes display especially low proportions of genes with low FPKMs. This leads to a higher transcript abundance of genes with low FPKMs. B) Comparison of the usage of 5' and 3' splice sites. On the x-axis, the difference between the 5' splice site usage and the usage of the 3' splice site is shown. A fast drop of values when going to the negative side of the x-axis indicates that the 3' splice site is probably more flexible than the 5' splice site.

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Introns are mostly defined by phylogenetically conserved splice sites, but nevertheless some variation of these splice sites is possible [9, 10, 25, 26, 40]. To understand the amount of flexibility in respect to different terminal dinucleotides, we compared the usage of donor and acceptor splice sites over 4,141,196 introns in plants, 3,915,559 introns in animals and 340,619 introns in fungi (Figure 4B). The plot shows that the 3' splice site seems to be more fexible than the 5' splice site which was observed in all three kingdoms. Our observations align well with previous findings of a higher flexibility at the 3' splice site compared to the 5' splice site. A mutated 5' splice site represses the removal of the upstream intron [10, 42, 43]. Further, for plants and animals, the difference between the usage of the 5' splice site and the 3' splice site is notably higher for introns with the splice site combination GC-AG.

Although *bona fide* non-canonical splice site combinations are present in many plant transcripts [10], additional isoforms of the genes might exist. To evaluate the relevance of such alternative isoforms, we assessed the contribution of isoforms to the overall abundance of transcripts of a gene. Therefore, the usage of splice sites flanking an intron was compared to the average usage of splice sites. This reveals how often a certain intron is removed by splicing. Introns with low usage values might only be involved in minor transcript isoforms. While most introns display no or very small differences, GT-AG introns deviate from this trend. This indicates that non-canonical splice site combinations are frequently part of the dominant isoform. Again, these findings were similar for all of the investigated kingdoms.

## Conclusion

Our investigation of non-canonical splice sites in animals, fungi and plants revealed kingdom specific differences. Animal species with a high proportion of GA-AG splice site combinations were examined. Further, properties

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of introns and splice sites were analysed. One aspect of this analysis is, that the 3' splice site seems to be more flexible than the 5' splice site, which was observed in all three kingdoms. In fungi, the splice site combination CT-AC is more frequent than the splice site combination AT-AC. This makes CT-AC a major non-canonical splice site combination in fungal species, while AT-AC should be considered a minor non-canonical splice site in fungi. Overall, our findings demonstrate the importance of considering non-canonical splice sites despite their low relative frequency in comparison to the canonical splice site combination GT-AG. RNA-Seq data confirmed the existence and usage of numerous non-canonical splice site combinations. By neglecting non-canonical splice sites, *bona fide* genes might be excluded or at least structurally altered.

# Methods

## Analysis and validation of splice site combinations

Genome sequences (FASTA) and corresponding annotations (GFF3) of 130 fungal species and 489 animal species were retrieved from the NCBI. Representative transcript and peptide sequences were extracted as described before [10]. General statistics were calculated using a Python script [10]. The completeness of all data sets was assessed with BUSCO v3 [44] using the reference data sets 'fungi odb9' and 'metazoa odb9', respectively [45] (AdditionalFile 14 and 15). To validate the detected splice site combinations, paired-end RNA-Seq data sets were retrieved from the Sequence Read Archive [46] (AdditionalFile 16 and 17). The following validation approach [10] utilized STAR v2.5.1b [47] for the read mapping and Python scripts for downstream processing (https://doi.org/10.5281/zenodo.2586989). An overview of the RNA-Seq read coverage depth of splice sites in animals [48] and fungi [49] is available. RNA-Seq read mappings with STAR and HiSat2 were compared based on a gold standard generated by exonerate, because a previ-

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ous report [50] indicated a superiority of STAR. All transcripts with non-canonical splice sites in *A. thaliana* and *Oryza sativa* were considered. When investigating the alignment of RNA-Seq reads over non-canonical splice sites, we observed a high accuracy for both mappers without a clear difference between them. Previously described scripts [10] were adjusted for this analysis and updated versions are available on github (https://doi.org/10.5281/zenodo.2586989). The distribution of genome sizes was analysed using the Python package dabest [51]. Sequence logos for the analysed splice sites were designed at `http://weblogo.berkeley.edu/logo.cgi` [52].

## Calculation of the splice site diversity

A custom Python script was applied to calculate the Shannon diversity index (H') [53] of all splice site combinations in fungi, animals and plants (https://doi.org/10.5281/zenodo.2586989). To determine the significance of the obtained results, a Kruskal-Wallis test [54] was calculated using the Python package scipy [55]. Further, the interquartile range of all distributions was examined.

## Investigation of a common non-canonical splice site in fungi

A Mann-Whitney U Test implemented in the Python package scipy was performed to analyse differences in the number of minor non-canonical splice site combinations. The observed distributions were visualized in a boxplot (https://doi.org/10.5281/zenodo.2586989) constructed with the Python package plotly [56].

## Detection of potential U1 snRNAs

A potential U1 snRNA of *Pan troglodytes* (obtained from the NCBI) was subjected to BLASTn [57] against the genome sequences of selected

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species. Hits with a score above 100, with at least 80 % similarity and with the conserved sequence at the 5' end of the snRNA [58] were investigated, as these sequences are potential U1 snRNAs. The obtained sequences were compared and small nucleotide variants were detected.

## Correlation between the GC content of the genome and the GC content of the splice sites

The Pearson correlation coefficient between the GC content of the genome sequence of each species and the GC content of the respective splice site combination was calculated using the Python package scipy. Splice site combinations were weighted with the number of occurences while calculating the GC content. Finally, the correlation coefficient and the p-value were determined. For better visualization, a scatter plot was constructed with the Python package plotly [56].

## Phylogeny of non-canonical splice sites

All *A. thaliana* transcripts with non-canonical splice sites were subjected to BLASTn searches against the transcript sequences of all other plant species previously studied [10]. The best hit per species was selected for an alignment against the respective genomic region with exonerate [59]. Next, splice site combinations were extracted and aligned. This alignment utilized MAFFT v7 [60] by representing different splice site combinations as amino acids. Finally, splice site combinations aligned with the non-canonical splice site combinations of *A. thaliana* were analysed (https://doi.org/10.5281/zenodo.2586989).

## Usage of non-canonical splice sites

Genes were classified based on the presence/absence of non-canonical splice combinations into four groups: GT-AG, GC-AG, AT-AC, and minor non-canonical splice site genes. When having different non-canonical

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splice sites, genes were assigned into multiple groups. Next, the transcription of these genes was quantified based on RNA-Seq using featureCounts [61] based on the RNA-Seq read mapping generated with STAR. Binning of the genes was performed based on the fragments per kilobase transcript length per million assigned reads (FPKMs). Despite various shortcomings [62], we consider FPKMs to be acceptable for this analysis. Outlier genes with extremely high values were excluded from this analysis and the visualization. Next, a cumulative sum of the relative bin sizes was calculated. The aim was to compare the transcriptional activity of genes with different splice site combinations i.e. to test whether non-canonical splice site combinations are enriched in lowly transcribed genes.

Usage of splice sites was calculated per intron as previously described [10]. The difference between both ends of an intron was calculated. The distribution of these differences per splice site type were analysed. Introns were grouped by their splice site combination. The average of both coverage values of the directly flanking exon positions was calculated as estimate of the local expression around a splice site combination. Next, the sequencing coverage of a transcript was estimated by multiplying 200 bp (assuming 2x100 nt reads) with the number of read counts per gene and normalization to the transcript length. The difference between both values was calculated for each intron to assess its presence in the major isoform.

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# Additional Files

**AdditionalFile 1.** List of genome sequences and annotations of the investigated animal species.

**AdditionalFile 2.** List of genome sequences and annotations of the investigated fungal species.

**AdditionalFile 3.** Distribution of genome sizes of all species.

**AdditionalFile 4.** Distribution of canonical and non-canonical splice sites per species in the animal kingdom.

**AdditionalFile 5.** Distribution of canonical and non-canonical splice sites per species in the fungal kingdom.

**AdditionalFile 6.** List of all possible splice site combinations in animal species.

**AdditionalFile 7.** List of all possible splice site combinations in fungal species.

**AdditionalFile 8.** Genome statistics concerning each analysed animal species.

**AdditionalFile 9.** Genome statistics concerning each analysed fungal species.

**AdditionalFile 10.** Correlation between the GC content of the genome and the GC content of the splice sites per kingdom.

**AdditionalFile 11.** Correlation between the size of the used RNA-Seq data sets and the number of supported splice sites.

**AdditionalFile 12.** Proportion of genes with non-canonical splice sites in dependence of the number of introns.

**AdditionalFile 13.** Usage of non-canonical splice sites in animals and fungi.

**AdditionalFile 14.** Non-canonical splice sites in BUSCOs and in all genes were assessed per species in the animal kingdom.

**AdditionalFile 15.** Non-canonical splice sites in BUSCOs and in all genes were assessed per species in the fungal kingdom.

**AdditionalFile 16.** List of Sequence Read Archive accession numbers of the investigated animal RNA-Seq data sets.

**AdditionalFile 17.** List of Sequence Read Archive accession numbers of the investigated fungal RNA-Seq data sets.