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CELLULAR AND MOLECULAR BIOLOGY

In silico analysis of *Apostasia wallichii* (Apostasioideae) and *Ludisia discolor* (Orchidoideae) orchids reveals different repeats composition despite the same genome size

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Abstract: Repetitive sequences can lead to variation in DNA quantity and composition among species. The Orchidaceae, the largest angiosperm family, is divided into five subfamilies, with Apostasioideae as the basal group and Orchidoideae and Epidendroideae showing high diversification rates. Despite their different evolutionary paths, some species in these groups have similar nuclear DNA content. This study focuses on one example to understand the dynamics of major repetitive DNAs in the nucleus. We used Next-Generation Sequencing (NGS) data from *Apostasia wallichii* (Apostasioideae) and *Ludisia discolor* (Orchidoideae) to identify and quantify the most abundant repeats. The repetitive fraction varied in abundance (27.5% in *L. discolor* and 60.6% in *A. wallichii*) and composition, with LTR retrotransposons of different lineages being the most abundant repeats in each species. Satellite DNAs showed varying organization and abundance. Despite the unbalanced ratio between single-copy and repetitive DNA sequences, the two species had the same genome size, possibly due to the elimination of non-essential genes. This phenomenon has been observed in other *Apostasia* and likely led to the proliferation of transposable elements in *A. wallichii*. Deep genome information in the future will aid in understanding the contraction/expansion of gene families and the evolution of sequences in these genomes.

Key words: Interspecific variation, Nuclei DNA, Orchidaceae, Repeat Explorer, retrotransposons, satellite DNA.

INTRODUCTION

The nucleus houses the genetic information, which is a combination of DNA and histone proteins that goes through various stages of compactation during the cell cycle (Pietro & Maeshima 2019). Overall, the amount of DNA in the nucleus shows no evolutionary correlation, the so-called C-value paradox, and variations in this feature have been linked to the amplification/removal of different classes of repetitive DNA within the cell (Eddy 2012).

This repetitive fraction can be divided into two main types, transposable elements, i.e. TEs (mainly DNA transposon and retrotransposon), and satellite DNAs (satDNAs) (Wells & Feschotte 2020, Thakur et al. 2021). The first class usually undergoes cycles of activation/inactivation through transposition/retrotransposition events that results in copy number changes and spatial reorganization of these elements along the genome (Wells & Feschotte 2020). As a result, DNA quantity (genome size) may increase or decrease between relatives (Macas et al. 2015, Galbraith et al. 2021), or even within populations of the same species (Lockton et al. 2008). Meanwhile, satDNAs may not be responsible for large-scale variation in genome size, but are

associated with rapid qualitative differences in repetitive composition (Belyayev et al. 2019, Palacios-Gimenez et al. 2020).

Orchidaceae is the largest family among flowering plants and includes more than 25,000 species distributed mainly in the tropical region of Asia and South America (especially in Brazil, Colombia, Ecuador and Peru; Dressler 2005). According to the current taxonomic knownledge, the family is divided into five subfamilies (Apostasioideae, Vanilloidieae, Cypripedioideae, Orchidoideae, and Epidendroideae) with distinct relationships. Apostasioideae, the earliest diverged group (90 Mya), consist of 14 species of two genera (*Apostasia* and *Neuwiedia*) that have retained several basal features in terms of morphology and pollination biology and is considered as a sister clade to all other orchids. The recently evolved groups Orchidoideae and Epidendroideae (64 Mya) comprise the largest number of living representatives and show the highest diversification rates within the family (Kocyan & Endress 2001, Givnish et al. 2015, Christenhusz et al. 2017).

The current genomic knowledge of orchids includes the assembly of ~120 plastomes of representatives from different subfamilies (see, for example, Kim et al. 2020), transcriptome data for around 100 species (reviewed by Wong & Peakall 2020) and whole-genome sequences for *Dendrobium catenatum* and *Phalaenopsis equestris* (Epidendroideae), *Apostasia shenzhenica* (*Apostasioideae*) and *Platanthera guangdongensis* and *P. zijinensis* (Orchidoideae) (reviewed by Chen et al. 2022). Together, these data have allowed the annotation of several genomes and have been used for phylogenomic studies in the family, contributing to a better understanding of the life history of Orchidaceae (Song et al. 2022).

However, none of these studies examined the diversity of repetitive sequences in detail and in a comparative/evolutionary pathway, as information on repetitive DNA is only available for the sequenced species. This nuclear fraction is directly related to transitions in genome composition and size. The latter feature shows a range of ~ 168- fold in the Orchidaceae, with a 1C value between 0.33 and 55.4 pg (Tsai et al. 2017). However, some species from unrelated groups still have the same genome size, such as *Apostasia wallichii* (Apostasioideae) and *Ludisia discolor* (Orchidoideae), whose haploid genome size is about 1.1 pg or 1075.8 Mbp (Jersáková et al. 2013, Trávníček et al. 2015).

In the present work, we have selected these aforementioned species, taking advantage of the genome skimming data available for both, to investigate the composition and abundance of repetitive DNA, using a computational clustering approach, and to understand how this fraction evolves in such a contrasting scenario where the same genome size has been maintained/achieved over the long evolutionary timespan of these subfamilies.

MATERIALS AND METHODS

Genomic data source and sequence editing

Raw genomic paired-end reads generated by Illumina sequencing and available for *Apostasia wallichii* (code SRX2338502) and *Ludisia discolor* (code SRX1747043) were retrieved from the Sequence Read Archive (SRA; (https://www.ncbi.nlm.nih.gov/sra). The sequences were then uploaded to the Repeat Explorer server (https://repeatexplorer-elixir.cerit-sc.cz/galaxy) and subjected to several processing steps following Novák et al. 2020. First, the reads were tagged with a specific code to correctly identify each species. Next, they were trimmed to a size of 200 bp and then filtered for quality using the following parameters: a quality cutoff of 10 with a percentage above the cutoff of 95 and exclusion

of those reads where N bases were found. In this way, the output file of each species was used to sample a set of 3000000 random reads and then concatenated in a single file for further study.

Repetitive DNA annotation and phylogenetic analysis

The RepeatExplorer 2 clustering tool, implemented on the same server, was used to identify and quantify the repetitive elements through a clustering approach with default parameters (Novák et al. 2020). The analysis was performed using both the concatenated dataset (for a comparative approach) and the individual sets of 3000000 reads (to confirm the major repeats in each genome). The output files (clusters with genomic abundance greater than 0.01%) were manually inspected and the proportion of each repetitive element was determined. For satDNAs, an additional analysis was conducted using the individual dataset of 3000000 reads of each species and the TAREAN (Tandem Repeat Analyzer) tool also implemented within the Repeat Explorer server. This strategy aimed to sample any satDNA that might be underrepresented in the clustering analysis. The identified sequences were then aligned with the clustering results to look for similarities between the already annotated sequences. Different characteristics of the satDNAs were also analyzed (size, sequence composition, genomic frequency) and similarity among them was checked by dotplot comparisons (Sonnhammer & Durbin 1995).

The phylogenetic analysis was conducted using the maximum likelihood method (RAxML) in Geneious v. 9.1.8. DNA sequences for the retrotranscriptase (RT) and RNase H (RH) domains (lineages Angela) as well as the integrase (INT; Tekay) were identified and extracted from the contigs. This was achieved by performing a homology survey against the Conserved Domain Database (CDD, NCBI). The nucleotide sequences for each domain were translated into all possible frames and aligned with a set of polyprotein domains (RT, RH or INT) from various plant species (Neumann et al. 2019) using Clustal W. The resulting tree topology was visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

A set of ~ 750000 reads from each species (corresponding to ~ 0.1 × genome coverage) was analyzed in the comparative clustering and revealed repetitive fractions of varying sizes (Table I). While *L. discolor* harbors ~ 27.5% of this type of sequences, they are more than twice as high in *A. wallichii* (60.6%). The two most abundant sequences were LTR retrotransposons (15.8% and 55.0%, respectively) and unclassified repeats (4.73% and 4.02, respectively), followed by satellite DNA (3.15%), pararetrovirus (2.71%) and rDNA (0.65%), in the case of *L. discolor*, or DNA transposon (0.65%), rDNA (0.58%) in addition to satDNAs (0.31%) for *A. wallichii* (Fig. 1a, Table II). The top-ranked repeats in both individual analyses were the same and had similar abundances (data not shown).

Among the LTR retrotransposons, the Ty3-Gypsy superfamily was particularly noticiable (9.39% and 31.36%, respectively) in the two orchids analyzed here and consisted of up to six different lineages, some of which were shared. The Tekay lineage made up 8.07% of *L. discolor*

Table I. The number of sampled reads included within clustering by Repeat Explorer and the corresponding genomic coverage in relation to genome sizes.

	L. discolor	*A. wallichii*
Number of used reads	745777	757468
Genome size (Mbp)	1078,5	1078,5
Genomic coverage (×)	0.139	0.141

and 14.37% of *A. wallichii*, while the Ogre lineage accounted for 0.37% and 13.21%, respectively. At the same time, Ty1-Copia elements were primarily composed of the SIRE lineage (4.48% of 6.44%) in *L. discolor* and the Angela lineage (12.55% of 17.77%) in *A. wallichii*, in addition to five others observed in either species (Figs. 1b, c, Table II).

Four satDNAs with varying size, nucleotide composition and genomic frequency were identified in the comparative analysis. *Ldi*SAT1 (1.78%) and *Ldi*SAT2 (1.37%) were unique to *L. discolor* (3.15%), while *Awa*SAT1 (0.14%) and *Awa*SAT2 (0.17%) were specific to *A. wallichii* (0.31%) (Supplementary Material - Table SI, Data SI). Individual analysis using the TAREAN tool also identified three additional sequences for *L. discolor* but they were not quantified in the genomes as they were not among the most abundant sequences (above 0.01%). All satDNAs were compared with each other and no significant similarity was found (data not shown).

Reads from a particular repetitive element were never shared, as shown in Figure 2. Phylogenetic analysis of the protein domains of the most widespread LTR retrotransposons Ty1-Copia (Angela) and Ty3-Gypsy (Tekay) LTR retrotransposons also confirmed this lack of similarity between species, with sequences of the same origin (species) always being more similar to each other than to their counterparts (data not shown).

DISCUSSION

This study provide the first in depth analysis of repetitive DNA in *Ludisia* and the third for *Apostasia*, enabling the accurate identification of the main repeats and their abundance in these species. Draft genomes of other orchids show repetitive fractions of different sizes, such as the two extreme examples of *Plalanthera* species (*P. guangdongensis* and *P. zijinensis*; Li et al. 2022), where the large-sized genomes (~ 4.24 pg and ~ 4.37 pg) are mainly composed of this type of sequences (77.38% and 82.18%, respectively; Li et al. 2022). In the case of *Apostasia*, the annotation of *A. shenzhenica* (Zhang et al. 2017) and *A. ramifera* (Zhang et al. 2021) revealed repetitive portions of around 42.05% and 44.99% each. These percentages are lower than what was found for *A. wallichii* in the present study, but they may be consistent with

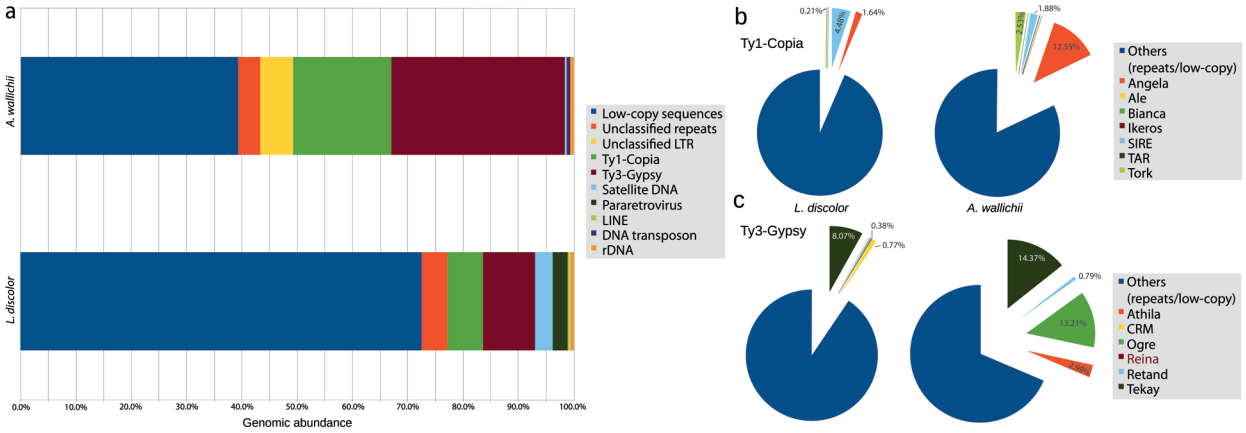

Figure 1. Overview of the repetitive landscape in the genomes of *Ludisia discolor* and *Apostasia wallichii* using Repeat Explorer. a) Relative abundance of the main classes of repetitive sequences in relation to the low-copy fraction. Comparative percentage of the most abundant LTR retrotransposons from lineages Ty1-Copia (b) and Ty3-Gypsy (c) between the two species.

Table II. Summary of the major repetitive elements found in the genome of *L. discolor* and *A. wallichii* orchids using a clustering-based approach.

Repetitive sequences	*L. discolor*	*A. wallichii*
Unclassified repeats	4.74	4.02
LTR retrotransposons		
Unclassified	0.00	5.92
Ty1-Copia		
Angela	1.64	12.55
Ale	0.00	0.18
Bianca	0.00	0.28
Ikeros	0.00	0.28
SIRE	4.48	1.88
TAR	0.12	0.09
Tork	0.21	2.53
Ty3-Gypsy		
Athila	0.00	2.99
CRM	0.77	0.00
Ogre	0.38	13.21
Reina	0.07	0.00
Retand	0.10	0.79
Tekay	8.07	14.37
Satellite DNA	3.15	0.31
Pararetrovirus	2.71	0.00
LINE	0.32	0.00
DNA transposon		
CACTA	0.00	0.61
hAT	0.02	0.05
Harbinger	0.02	0.00
rDNA		
5S	0.04	0.08
35S	0.62	0.50
Total repetitive	27.47	60.64

the smaller genome sizes of these species (0.4 pg in *A. shenzhenica* and 0.34 pg in *A. ramifera*; Jersáková et al. 2013, Zhang et al. 2021).

LTR-like sequences are highly prevalent in plant genomes, including all orchids studied so far either by deep genome annotation (see previous references) or with tools such as Repeat Explorer (e.g. *Paphiopedilum* Pfitzer, Cypripedioideae; Lee et al. 2018). Aditionally, these sequences are strongly linked to alteration in genome composition and size (Ramakrishnan et al. 2022). Overall, our clustering analysis showed that, except for rDNA, reads of the same element did not group together if they were from different species. This was also observed when a phylogenetic analysis was performed. Thus, at the nucleotide level, a common transposable element is similar enough to maintain the same nomenclature but different enough to prevent it from being clustered together when present in both *L. discolor* and *A. wallichii*.

The annotation strategy used for identifying repeats in *A. shenzhenica*, *A. ramifera* (Zhang et al. 2017, 2021) and other draft genomes of orchids did not distinguish lineages of LTR retroelements, making it difficult to compare *L. discolor* and *A. wallichii* to those species. However, Ogre elements were found in a significant amount (30-46%) in all nine species of lady slipper orchids (*Paphiopedilum*) analyzed by Lee et al. 2018, representing the majority of the repetitive fraction. These elements, along with Tekay-like or Angela-like LTR retrotransposons, are also known to be highly abundant in grasses (Amosova et al. 2022, Moreno-Aguilar et al. 2022) and legumes (Macas et al. 2015).

Although not as diverse in the number of sequences as in other plant species (e.g. Amosova et al. 2022), the satellitome of *L. discolor* and *A. wallichii* varied by a 10-fold difference. None of these satDNAs showed significant similarity to each other, suggesting that each species possessed a specific library of satDNAs, likely arisen after the divergence of the subfamily Apostasioideae (around 90 Mya, Givnish et al. 2015). In this scenario, these sequences could only be shared between closely related species or genera, so further analysis of other *Ludisia* and *Apostasia* is needed to fully understand the satDNA landscape.

Despite differences in sequence composition and the ratio between low-copy and repetitive

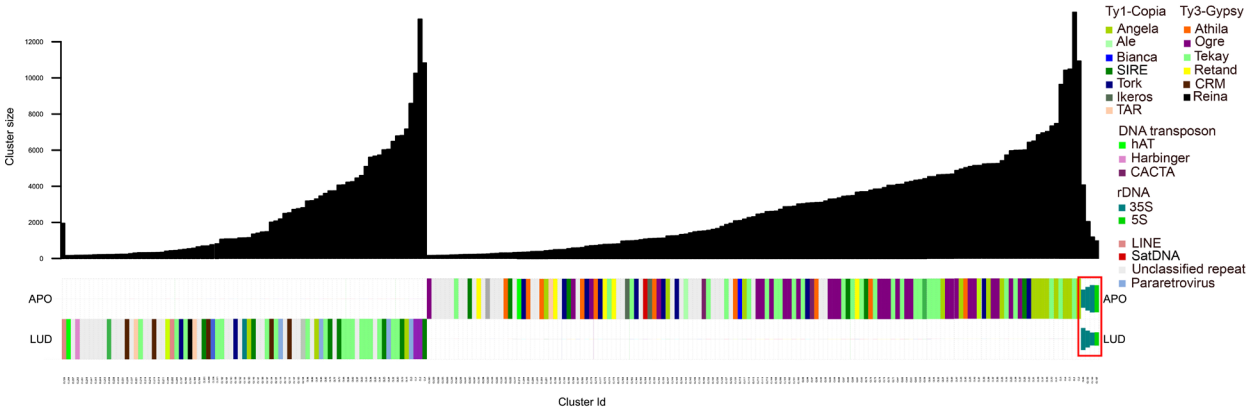

Figure 2. Clusters of *L. discolor* (LUD) and *A. wallichii* (APO) identified by the Repeat Explorer in relation to the abundance of reads (cluster size, upper panel) and sequence identity (colored bars in the lower panel). The height of the bar in the upper part represents the quantity of reads in a specific cluster, while the color in the lower panel points to the type of sequence in that cluster (See legend for details). Sequences were almost exclusive to each species, except for those clusters belonging to the rDNA (red box in the lower right side).

sequences, *L. discolor* and *A. wallichii* have similar genome sizes (Jersáková et al. 2013, Trávníček et al. 2015). Analyses in Orchidaceae have shown that changes in gene, such as gene loss, pseudogenization, and reductions in the number of introns and average gene length, may be linked to the rapid radiation observed in several family groups (e.g. Zhang et al. 2016). For instance, gene family shrinkage seems to be more common than expansion in species of *Apostasia* (*A. shenzhenica* and *A. ramifera*, Zhang et al. 2017, 2021) and is also observed in *Plalanthera* (Orchidoideae) genomes. The latter genus shows high rates of gene loss and the spread of transposable elements, attributed to a shift from autotrophism to mycoheterotrophism, allowing for the replacement of obsolete genes with repetitive DNA (Li et al. 2022). *Apostasia wallichii* is an initially mycoheterotrophic species and belongs to a genus where gene loss has been reported (Zhang et al. 2017, 2021). Therefore, the increase in repetitive content without affecting genome size may have been linked to the previous deletion of non-essential genes making room for the amplification of repetitive sequences.

CONCLUSIONS

Apostasia wallichii and *L. discolor* exhibited significant differences in the amount and composition of their repetitive DNA fraction, despite having similar nuclear genome sizes. Transposable elements were the primary component in both species, represented by some prominent lineages of LTR retrotransposon. It is likely that these elements increased in percentage in *A. wallichii* occupying the space created by the removal of non-functional genes. The availability of draft genomes for both species in the near future will provide an opportunity to analyze the evolution of the gene families and test for these potential expansion and/or contraction events.

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SUPPLEMENTARY MATERIAL

Table SI.

Data SI.

How to cite

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