The first genome for the Cape Primrose *Streptocarpus rexii* (Gesneriaceae), a model plant for studying meristem-driven shoot diversity

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**Abstract**
Cape Primroses (*Streptocarpus*, Gesneriaceae) are an ideal study system for investigating the genetics underlying species diversity in angiosperms. *Streptocarpus rexii* has served as a model species for plant developmental research for over five decades due to its unusual extended meristem activity present in the leaves. In this study, we sequenced and assembled the complete nuclear, chloroplast, and mitochondrial genomes of *S. rexii* using Oxford Nanopore Technologies long read sequencing. Two flow cells of PromethION sequencing resulted in 32 billion reads and were sufficient to generate a draft assembly including the chloroplast, mitochondrial and nuclear genomes, spanning 776 Mbp. The final nuclear genome assembly contained 5,855 contigs, spanning 766 Mbp of the 929-Mbp haploid genome with an N50 of 3.7 Mbp and an L50 of 57 contigs. Over 70% of the draft genome was identified as repeats. A genome repeat library of Gesneriaceae was generated and used for genome annotation, with a total of 45,045 genes annotated in the *S. rexii* genome. Ks plots of the paranomes suggested a recent whole genome duplication event, shared between *S. rexii* and *Primulina huaijiensis*. A new chloroplast and mitochondrial genome assembly method, based on contig coverage and identification, was developed, and successfully used to assemble both organellar genomes of *S. rexii*. This method was developed into a pipeline and proved widely applicable. The nuclear genome of *S. rexii* and other datasets generated and reported here will be invaluable resources for further research to aid in the identification of genes involved in morphological variation underpinning plant diversification.

**KEYWORDS**
genome assembly, Gesneriaceae, high-molecular weight DNA, Oxford Nanopore Technologies, PLCL pipeline, *Streptocarpus rexii*
Biodiversity greatly reflects the genetic properties of organisms (e.g. Supple & Shapiro, 2018). Unraveling the genes underlying this diversity is an ongoing challenge in evolutionary research. Recent advances in sequencing technologies made the acquisition of whole genome data rapid and affordable even for non-model plants (e.g., Dumschott et al., 2020; Liu et al., 2019). In this study, we use Oxford Nanopore Technologies (ONT) long read sequencing (Rang et al., 2018) to assemble the nuclear, chloroplast, and mitochondrial genomes of the emerging model plant species, the Cape Primrose Streptocarpus rexii (Bowie ex Hook.) Lindl. The plants’ haploid nuclear genome size was previously estimated with flow cytometry to be around 929 Mb, falling within the average range of angiosperm genome sizes (Dodsworth et al., 2015). It has a karyotype of $n = x = 16, 2n = 32$ chromosomes (Möller, 2018) (Figure 1).

Streptocarpus rexii belongs to a genus of the Old World (OW) members of the plant family Gesneriaceae (Weber et al., 2013). Streptocarpus includes 185 species (Hilliard & Burtt, 1971; Nishii et al., 2015; GRC, 2021 onwards, https://padme.rbge.org.uk/grc), and some of these are popular ornamental plants. Streptocarpus species are particularly notable for their vegetative and floral diversity (e.g., Hilliard & Burtt, 1971; Nishii et al., 2015). The flowers are zygomorphic and possess a variety of shapes and colors and markings representing different pollination syndromes (Möller et al., 2019).

Besides their ornamental value, Streptocarpus are historically of great interest to evolutionary biologist (Crocker, 1861), because of their unique vegetative forms, which develop from uniquely evolved and behaving leaf and shoot apical meristems (Jong, 1970; Jong & Burtt, 1975; Steeves & Sussex, 1989). The roles of shoot apical meristems have partly been transferred to leaf meristems positioned at the proximal end of the leaf. These leaf meristems first appear in the cotyledons and their differential growth leads to asymmetrical laminal growth, forming a macrocotyledon and microcotyledon (Jong & Burtt, 1975; Imaichi et al., 2000; Nishii et al., 2004). Although cauline meristem species retain the shoot apical meristem and a short-lived leaf meristem for lamina expansion, unifoliate species only retain the macrocotyledon enlarging from a persistent basal leaf meristem and are the sole photosynthesizing foliar organ. They produce inflorescences from the base of the lamina of the macrocotyledon and are monocarpic. Some species produce additional leaves from the base of the macrocotyledon, which is arranged in a false rosette to produce the rosulate form such as found in S. rexii (Jong & Burtt, 1975; Möller & Cronk, 2001; Nishii & Nagata, 2007).

Streptocarpus rexii has been extensively studied as a model species in the genus, in particular from an evolutionary developmental point of view using candidate gene approaches. The roles of a few key meristematic genes characterized in model plants have been investigated in leaf meristems of S. rexii. These studies have shown that genes expressed in the shoot apical meristem of model plants, such as class 1 KNOX genes, WUSCHEL, ARP, and YABBY, are also expressed in the leaf meristems of Streptocarpus (Mantegazza et al., 2007, 2009; Nishii et al., 2010, 2017; Tononi et al., 2010). Thus, it seems that Streptocarpus has evolved unique genetic cascades, perhaps through novel genes or pathways to achieve its varied vegetative forms. The genetic differences between these forms appear to have relatively simple mechanisms, as shown in early traditional genetic studies. For example, Beuttel (1939) and Oehlkers (1964) independently studied the vegetative morphology of Streptocarpus and reported the involvement of one or two loci differentiating unifoliate and rosulate forms.

Recently, based on restriction site-associated DNA sequencing (RAD-seq), a genetic map was generated for Streptocarpus (Chen et al., 2018), and a QTL study on flower morphology reported (Chen et al., 2020). The actual key genes regulating vegetative or floral
morphologies are yet to be discovered, and a nuclear genome sequence for Streptocarpus would open the door to discover these genes, as well as lineage specific genetic events involved in their evolution.

The unique meristem properties of S. rexii mean that newly generated genomic resources may facilitate critical insights into the genetic mechanism underlying meristem establishment in plants. Some genetic resources are already available for the genus, such as a transcriptome (Chia et al., 2013), and an interspecific genetic map using a cross between the rosulate S. rexii and the unifoliate S. grandis (Chen et al., 2018). These resources, together with the nuclear genome assembled here would aid studies to pinpoint the

20 species including 11 species of Primulina and OW Gesneriaceae. In total, transcriptomes are available for OW Gesneriaceae, but none for New World (NW) taxa. Nuclear genome assemblies exist for Doroceras hygrometricum (formerly Boea hygrometrica) (Xiao et al., 2015) and Primulina huaijiensis (Feng et al., 2020) (Table S1). There are several intriguing developmental and ecological features of these two taxa: for example, D. hygrometricum was studied for its desiccation tolerance (Mitra et al., 2013), and a genome-wide DNA methylation study suggested a rapid genome-wide response upon desiccation (Sun et al., 2021). Primulina, on the other hand, was studied intensively at a genus level from an ecological and systematic point of view, as well as for the development and diversity of flowers, for which a genetic map had been built (Feng et al., 2016, 2019, 2020).

Several transcriptome studies have been published for both NW and OW Gesneriaceae. In total, transcriptomes are available for 20 species including 11 species of Primulina (Table S1). Raw transcriptome reads of S. ionanthus are deposited in the data archive of the 1kp project (One Thousand Plant Transcriptomes Initiative, 2019; Table 1). Those genome and transcriptome resources are investigated and incorporated into the annotation pipeline of S. rexii here.

In plant cells, DNA is found in the nucleus, the chloroplast, and the mitochondrion. In Gesneriaceae, chloroplast genomes of 18 species have been published at present, including two species and five subspecies of Streptocarpus (Table S2), whereas only one mitochondrial sequence is available, for D. hygrometricum (Zhang et al., 2012). In the present study, both chloroplast and mitochondrial genome sequences were assembled de novo for S. rexii using coverage information from the draft genome assemblies. This plastid assembly method was further developed into a pipeline and tested in Arabidopsis thaliana and Oryza sativa. This method neither requires a reference nor seed sequences, unlike many other methods (e.g., NOVOPlasty, Dierckxens et al., 2017; GetOrganelle, Jin et al., 2020). Unlike Organelle_PBA (Soorni et al., 2017), it does not require a scaffolding step. The method is also computationally efficient because it does not deal with all raw reads, but only those from the assembled contigs.

Although the method for whole genome sequencing and assembly are well established for model species (e.g., Dumschott et al., 2020; Michael et al., 2018), there are still technical challenges for successful genome sequencing. These include issues around the extraction of high-molecular weight, high-quality DNA, and the method developed here may prove to be widely applicable. As additional resources for OW Gesneriaceae, we generated a genome annotation pipeline. This study provides the resources and methodological approaches developed and applied here.

| Parameter                        | Values    |
|----------------------------------|-----------|
| Estimated genome size (Mb)a      | 929       |
| Assembled genome size (Mb)       | 766       |
| Num. of total contigs            | 5,855     |
| Num. of contigs ≥ 50 Kbp         | 1,811     |
| Longest contig (bp)              | 15,643,668|
| Statistics (≥3,000 bp)           |           |
| GC (%)                           | 38.89     |
| N50 (bp)                         | 3,726,467 |
| N75 (bp)                         | 1,476,021 |
| L50                              | 57        |
| L75                              | 135       |
| BUSCO completeness (%)           | 99.0      |
| Genome repeats (%)               | 70.97     |
| Num. genes annotated             | 45,045    |
| Average gene length (bp)         | 2,609     |
| Num. of exons                    | 213,819   |
| Average num. exons per gene      | 4.7       |
| Average exon length (bp)         | 249       |
| BUSCO completeness of annotated gene set (%) | 89.6  |
| Chloroplast genome length (bp)   | 152,571   |
| Mitochondrial genome length (bp) | 599,262   |

aBased on Möller (2018).
2 | MATERIALS AND METHODS

2.1 | Plant material

*Streptocarpus rexii* (RBGE accession numbers: 20180766, descendent of lineage 19870333; K.Jong-Faraway) was in cultivation at the living collection of Royal Botanic Garden Edinburgh (RBGE). RBGE has maintained an inbred lineage of this species (Hughes et al., 2005) by self-fertilization of individuals over more than 10 generations (Chen et al., 2018).

2.2 | Metaphase and karyotype of *S. rexii*

The chromosome preparation followed Jong and Möller (2000). Images were captured with a Zeiss Zen v.3.1 and an AxioCam MRC5 camera mounted on an Axiohot brightfield microscope (Zeiss, Welwyn Garden City, UK). Images were manipulated in Gimp v.2.10.24 (The GIMP Development Team, 2019), chromosomes measured with Zen, and the karyotype created manually in Powerpoint (Microsoft Office).

2.3 | DNA extraction

We established a reliable protocol for extracting high-quality, high-molecular weight plant DNA. To obtain such DNA requires careful consideration of the plant material and extraction methods; for example, the leaf material is best used fresh, not silica-dried; high temperatures should be avoided during extraction to keep damage to the DNA at a minimum. In the preliminary stage, we tested “traditional” methods such as CTAB (Doyle, 1991; Doyle & Doyle, 1987), Qiagen DNeasy kit (Hilden, Germany), and the Invitrogen PureLink™ Genomic Plant DNA kit (Waltham, MA, US), but these did not generate sufficiently high-quality DNA for ONT sequencing (data not shown). Thus, we developed a DNA extraction protocol based on those reported by Souza et al. (2012), Gunter (2015), and PacBio Sample Net (2015), combining a nuclear isolation method, sorbitol buffer wash, and Qiagen Genomic tip columns. The detailed DNA extraction protocol has been deposited at protocol.io (dx.doi.org/10.17504/protocols.io.bempj5n) and is also available at RBGE’s web magazine (https://stories.rbge.org.uk/archives/30792).

2.4 | Library preparation and sequencing

Two libraries (11843TA0001L01 and 11843TA0001L02) were prepared with different shearing settings of 50 and 45 kbp, using a MegaRuptor (Diagenode, Denville, NJ, US). Smaller fragments of approximately less than 1 kbp were removed with AMPure XP beads (Beckman Coulter, Brea, CA, US) with a beads: sample ratio of 0.4 : 1 (v : v). For each library, 1 μg of sheared and cleaned DNA was used. Formalin-fixed, paraffin-embedded (FFPE) DNA repair reactions using NEBNext™ FFPE DNA Repair Mix (New England Biolabs, Ipswich, MA, US), end repair/A tailing using NEBNext™ Ultra End Repair/dA-Tailing Module (NEB) were carried out, and the samples were re-cleaned with AMPure XP beads (beads: sample ratio of 0.4 : 1 (v : v)). Adapter ligation was carried out using the Ligation Sequencing SQK-LSK109 kit (ONT, Littlemore, Oxford, UK), and the samples were further cleaned with the same ratio of AMPure XP beads to reduce fragment size to <3 kb. The long fragment buffer (LFB, a component of the SQK-LSK109 kit) was used as bead wash buffer. *Escherichia coli* DNA was added as a spiked-in control to verify the quality of the library preparation and sequencing. The two libraries were quantified and were 16.4 and 18.5 fmol, respectively. They were loaded separately on a PromethION Flow cell FLO-PRO002 (ONT). The sequencing run was set for 63 h, with a pore type: R9.4.1, and caller variant set to “fast.” Basecalling was done with Guppy v.3.0.5 (ONT).

2.5 | Read quality control

The quality of reads and the read lengths were assessed with NanoPlot v.1.38.0 (De Coster et al., 2018), and the summary text files provided by Guppy were examined. The read length versus read number histogram plots were generated using R ggplot2 (Wickham, 2016) with a bin size of 1,000.

2.6 | Genome assembly

Two fastq read sets generated from the two sequencing libraries were combined into one file and the assembly performed with Canu v.1.8 (Koren et al., 2017). Only reads longer than 10,000 bp were used for genome assembly. The draft assembly was further polished five times with Racon v.1.4.11 (Vaser et al., 2017). The polished assembly was further examined with Medaka v.0.11.5 (https://nanoporetech.github.io/medaka/) to resolve unreliable scaffolds. To check and remove contaminants from non-plant organisms, the assembly was analyzed with BlobTools v.1.1.1 (Laetsch & Blaxter, 2017), and only scaffolds identified as streptophyta or no-hit were kept in the final assembly.

2.7 | Preparation of Gesneriaceae genome annotation materials

To annotate the *S. rexii* genome assembly produced in this study, we generated annotation material using resources available from public repositories (Table S12). The transcriptomes and proteomes of OW Gesneriaceae were obtained and curated for the genome annotation pipeline. The transcriptome of *S. rexii* was downloaded from Angeldust (http://www.beaconlab.it/angeldust) and their CDS predicted using TransDecoder v.5.5.0 (https://github.com/TransDecoder/TransDecoder). Transcriptomes were assembled de novo for *S. (Saintpaulia) ionantha* and *Haberlea rhodopensis* from raw reads (Table 3). The raw reads were trimmed using Trimmomatic v.0.39
(Bolger et al., 2014) and assembled using Trinity v.2.11.0 (Grabherr et al., 2011). For P. huaijiensis, the assembled transcriptome was obtained from Dryad (https://doi.org/10.1111/1755-0998.12333; Ai et al., 2015), and CDS and predicted protein sequences obtained using TransDecoder.

2.8 | Building Gesneriaceae genome repeat libraries

In the family Gesneriaceae, the genome assemblies of D. hygrometricum (previously Boea hygrometrica; Xiao et al., 2015) and P. huaijiensis (Feng et al., 2020) have been published and deposited in public databases. Genome repeat libraries were built from these assemblies and a S. rexii assembly generated in this study using the pipeline of Jacques Dainat (https://www.biostars.org/p/411101/). In brief, the de novo repeat libraries were built using RepeatModeler v.2.0 (Flynn et al., 2020). The same species’ proteomes were curated with TransposonPsi (http://transposonpsi.sourceforge.net/) to detect protein sequences of transposons, and the detected transposable element (TE) proteins removed from the proteome using the perl script “fasta_removeSeqFromDlList.pl” from the Genome Assembly Annotation Service (GAAS: https://github.com/NBISweden/GAAS). Blastp searches were carried out using the proteome without TE proteins as query and the generated repeat libraries as reference sequences. The resulting hits (sequences) were removed from the repeat libraries with ProtExcluder (https://github.com/NBISweden/ProtExcluder). This process was carried out for each species separately, with the resulting libraries concatenated and used as a Gesneriaceae genome repeat library for annotation.

2.9 | Genome repeat analyses

The genome repeats (repeated DNA sequences) in the S. rexii genome assembly were analyzed using RepeatMasker (Smit et al., 2013–2015; http://www.repeatmasker.org). The Gesneriaceae repeat libraries generated above, Dfam library (release 3.3, curated families) (Storer et al., 2021), and RepBase library (https://www.girinst.org/; RepBaseRepeatMaskerEdition-20181026) were concatenated and used with RepeatMasker.

2.10 | Genome annotation of S. rexii

The S. rexii genome was annotated with Maker (Holt & Yandell, 2011). The transcriptomes and proteomes of S. rexii and the other OW Gesneriaceae (P. huaijiensis, D. hygrometricum, S. ionanthus, H. rhodopensis), and the gff3 output of RepeatMasker, were used in the pipeline. After the first round of Maker carried out with the predicted gene models. In addition to the gff3 statistics in the Maker pipeline, Busco analyses were carried out. These, with the CDS sets of the nuclear genome annotated by Maker, were used to evaluate annotation efficiency.

2.11 | WGD analyses

WGD analyses were carried out on three Gesneriaceae species, S. rexii, P. huaijiensis, and D. hygrometricum using the program wgd (Zwaenepoel & van de Peer, 2019). To obtain CDS files mapped on the genome, the genome sequences of P. huaijiensis and D. hygrometricum were annotated using Maker, as for S. rexii described above, and gff3 files obtained. CDS files were generated from the gff3 files. For D. hygrometricum, the public CDS file (PRJNA182117, GCA_001598015.1_Boea_hygrometrica.v1_cds_from_genomic.fna.gz) was also analyzed, although not all sequences in the CDS assembly were assigned to a genome location. Orthologous pairs were found within and between species using wgd-mcl (Altschul et al., 1997), and synonymous substitution ratio (Ks) values were calculated using wgd-ksd with PAML4 (Yang, 2007) and MAFFT (Katoh & Standley, 2013). Co-linearity analyses were carried out using wgd-syn with i-ADHoRe (Proost et al., 2012). These analyses identified the anchor gene pairs, which are specific orthologous pairs arranged in the same order in a genome before and after a predicted WGD. Bayesian Gaussian mixture model (BGMM) analyses were carried out on the calculated Ks values using wgd-mix and visualized using wgd-viz in wgd.

2.12 | Chloroplast and mitochondrial genome assemblies

We tested two approaches for the assembly of chloroplast and mitochondrial genomes. One approach was to screen all raw reads using plastid queries as described in Wang et al. (2018), but this did not produce complete genomes for S. rexii; therefore, another approach was tested: The chloroplast and mitochondrial contigs were selected from the final whole genome assembly, the reads for these contigs extracted, and the chloroplast and mitochondrial genomes newly assembled. This strategy worked well for the S. rexii chloroplast and mitochondrial genome assemblies.

The draft genome scaffolds were analyzed with blastn (NCBI BLAST+ v.2.10.0; Altschul et al., 1994), against A. thaliana and Nicotiana tabacum chloroplast and mitochondrial genomes. The coverage of each scaffold was evaluated with BlobTools carried out as described above. The blastn results were compared against read coverage data. The input reads, that is, reads > 10,000 bp, were mapped onto the assemblies using minimap2 v.2.17 (Li, 2018) to generate a bamfile. From the bamfile, the mapped reads of chloroplast and mitochondrial contigs were extracted using samtools v.1.9 (Li et al., 2009).
The extracted chloroplast and mitochondrial reads were assembled using Canu. Several settings were tested, and the final settings used for the chloroplast assembly were minReadLength = 12,000, minOverlapLength = 10,000, corMinCoverage = 8, trimReadsOverlap = 100, and trimReadsCoverage = 100, and for the mitochondrial assembly corOutCoverage = 999, minReadLength = 10,000, minOverlapLength = 500, trimReadsOverlap = 3, and trimReadsCoverage = 3.

The chloroplast and mitochondrial sequences were annotated using GeSeq (Tillich et al., 2017). To compare the chloroplast sequences, first S. rexii, D. hygrometricum, and P. huaijiensis sequences were aligned using MAFFT and manually checked with BioEdit (Hall, 1999). The similarity of the sequences was analyzed in the mVISTA genome browser (Frazier et al., 2004) with the LAGAN aligner (Brudno et al., 2003). To analyze the similarity of the mitochondrial genomes between S. rexii and D. hygrometricum and also A. thaliana and the closely related Capsella rubella, the sequences were aligned one to one, and dot plots generated using the D-GENIES web server (Cabanettes & Klopp, 2018). Sequence accession numbers are shown in Table S13. Prior to the final nuclear genome assembly, chloroplast and mitochondrial contigs were removed.

### 2.13 PLCL pipeline

The approach used for the assembly of the S. rexii chloroplast and mitochondrial genomes was further developed into a pipeline, including perl scripts. The pipeline, named the plant contig clustering-based genome assembly (PLCL) pipeline, was subsequently tested with data from two representative model plants.

The PLCL pipeline was tested with A. thaliana KBS-Mac-74 ONT reads (ERR2173373; Michael et al., 2018) and O. sativa subsp. indica IR64 ONT reads (DRR196680; Tanaka et al., 2020). For each species, the ONT reads were evaluated using NanoPlot, and draft genomes assembled using Wtdbg2 (Ruan & Li, 2020). The resulting genome assembly contigs were blastn searched with chloroplast and mitochondrial sequences of each species as the query. To assess the contig coverage, the ONT reads were re-mapped onto the draft genome assembly using minimap2. The coverage was calculated using samtools. The results of blastn and coverage data were combined and the candidate plastid contigs selected. The contig coverage data were also assessed with the perl program Algorithm-KMeans-2.05 (https://metacpan.org/pod/Algorithm::KMeans) for rough clustering of plastid and nuclear contigs. The coverage and blastn bitscore datasets were manually checked to select chloroplast and mitochondrial contig candidates. The reads were extracted from bamfiles for each contig and assembled using Canu with different parameter settings (see Section 3 for details). The scripts of the PLCL pipelines have been deposited in protocol.io (dx.doi.org/10.17504/protocols.io.bx5dpq26).

### RESULTS

#### 3.1 ONT PromethION sequencing

The sequencing of libraries 11843TA0001L01 and 11843TA0001L02 generated 69.55 and 89.14 Gbp of data respectively, with >2,600 channels active in the flow cell during data production (Table S3). In total, we obtained 158.69 Gbp, in >32 billion reads of raw data. The read N50 was 10,281 and 18,877 bp, respectively. The longest read was >1.1 Mbp, in library 11843TA0001L02. This library generally generated longer read lengths (see Table S3) and a higher yield compared with library 11843TA0001L01. The majority of reads for both libraries ranged between 100 and 100,000 bp. The spike observed at around 3,500 bp in both libraries originated from the internal standard E. coli.

#### 3.2 Genome assembly

Only reads longer than 10,000 bp, that is, 4,360,109 raw reads of 89.56 Gbp combined from both libraries, were used for the genome assembly. These provided approximately 119 times coverage based on the estimated genome size of 929 Mb for S. rexii for the x = 16 chromosomes (Möller, 2018). The Canu analysis initially resulted in 2,488 contigs. While polishing the assembly, Racon reduced the number of contigs to 2,483, whereas Medaka dissolved uncertain contigs and resulted in a total of 6,155 contigs. The longest contig was 15.64 Mbp and the genome N50 was 3.6 Mbp (Table S4). Of the 6,114 contigs (776,293,364 bp) of >100 bp length, 2,035 contigs (96% of total bp) were assigned to streptophyta and 3,788 (2.75%) were no-hits (Figure S2; Table S5). The average coverage was 42.3× for streptophyta, 31× for no hit, and 17.2× for all contigs. Among the detected contaminants, the highest by base pairs was from proteobacteria (0.76%). All streptophyta (2.037) and no-hit (3.827) contigs, in total 5,864, were kept. Nine contigs were removed since they were assigned to the chloroplast and/or mitochondrial genomes (see below Section 3.5 for details), resulting in 5,855 nuclear contigs spanning 766 Mbp (Table 1, Table S7). The resulting nuclear genome assembly showed good contiguity, that is, 75% of the genome was covered by 135 contigs of >1 Mbp (see N75 and L75 in Table 1). The BUSCO completeness of the genome assembly against viridiplantae_odb10 was, at 99%, very high (Table 1, Table S6).

#### 3.3 Nuclear genome annotation

In the assembled S. rexii nuclear genome, 543,533,959 bp (70.97%) were identified as repeats (Table 1, Table S7). The retrotransposon portion was the highest among repeat elements (37.97%) (Table S8). In particular LTR retrotransposons occupied a large part of the genome space, at 35.29%, in which Gypsy/DIRS1 repeats were most abundant (22.05%), followed by Ty1/Copia (8.81%).
contrast, DNA transposons occupied only 1.15% of the genome. Unclassified repeats made up almost a third (31.05%) of the genome space.

With the available Gesneriaceae annotation resources (Table 2), Maker annotated 45,045 genes with an average gene length of 2,609 bp (Table 1). The BUSCO completeness of the genome annotation derived transcriptome was 89.6% (Table 1, Table S6).

### WGD analysis

WGD events were estimated for the *S. rexii* nuclear genome in comparison to those of *D. hygrometricum* and *P. huaijiensis* (Table 3). Prior to the WGD analyses, to obtain genome annotated CDS sets, the genomes of *D. hygrometricum* and *P. huaijiensis* were re-annotated in this study using the Maker pipeline, resulting in 24,585 gene models annotated in the *D. hygrometricum* genome and 42,685 in the *P. huaijiensis* genome.

For the paranomes, defined as the complete set of all duplicated genes in a genome (Vandepoele et al., 2004), synonymous substitution (Ks) plots were obtained for all candidate gene pairs and those of anchor gene pairs. For *S. rexii*, *P. huaijiensis*, and *D. hygrometricum*, 84,617, 49,048, and 56,877 whole paranome candidate gene pairs and 20,197, 9,919, and 760 anchor gene pairs were obtained respectively, indicating that the number of anchor gene pairs in *D. hygrometricum* was substantially lower than in the other two species (Figure 2; Table 3).

The Ks plots for *S. rexii* and *P. huaijiensis* were very similar, with a peak observed at \( K_S = 0.19 \) for both species for all paranomes and for anchor gene pairs (Figure 2). In contrast, the whole paranome of *D. hygrometricum* showed a first peak at \( K_S = 0.06 \) and a second broader peak around \( K_S = 1.0 \), but the first peak was not recognized with the anchor gene pairs. A fitted mixture model (BGMM) analysis also showed the mismatch between the Ks plots of whole paranomes and those anchor gene pairs for *D. hygrometricum* (Figure S3).

### TABLE 2 Gesneriaceae annotation resources used in the Marker annotation pipeline

| Type of resource       | Taxon                      | Reference                        |
|------------------------|----------------------------|----------------------------------|
| Genome repeat library  | Doroceras hygrometricum    | This study<sup>a</sup>           |
|                        | Primulina huaijiensis      | This study<sup>a</sup>           |
|                        | Streptocarpus rexii        | This study                       |
| Transcriptome          | *D. hygrometricum*         | Xiao et al. (2015)               |
|                        | Haberlea rhodopensis       | This study<sup>b</sup>           |
|                        | *P. huaijiensis*           | Ai et al. (2015)                 |
|                        | Streptocarpus ionanthus    | This study<sup>b</sup>           |
|                        | *S. rexii*                 | Chiara et al. (2013)             |

<sup>a</sup>Genome sequences obtained from NCBI.
<sup>b</sup>Reads were obtained from SRA archive.

### TABLE 3 Statistics of assembled genomes available for Gesneriaceae

|                      | *Streptocarpus rexii* | *Primulina huaijiensis* | *Doroceras hygrometricum* |
|----------------------|-----------------------|-------------------------|---------------------------|
| Estimated genome size (Mb) | 929                  | 511                     | 1,691 (240<sup>a</sup>)  |
| Assembled genome size (Mb) | 766                  | 478                     | 1,548                     |
| Num. of haploid chromosomes | \( n = 16 \)       | \( n = 18 \)            | \( n = 9<sup>b</sup> \)  |
| N50 (bp)              | 3,726,469             | 23,479,473              | 110,988                   |
| L50                   | 57                    | 9                       | 3,003                     |
| Genome repeats (%)    | 70.95                 | 54.10                   | 75.16                     |
| Num. of nuclear scaffolds | 5,855                | 18                      | 520,969                   |
| Num. of annotated genes (this study) | 45,045               | 42,685                  | 24,585                    |
| Num. of annotated genes (references) | —                   | 31,328                  | 23,250<sup>c</sup> (49,374<sup>d</sup>) |
| Total candidate gene pairs (whole paranome gene pairs) | 84,617               | 49,048                  | 56,877                    |
| Anchor gene pairs     | 20,197                | 9,919                   | 760                       |
| Orthologous gene pairs 1 | 12,160               | 12,160                  | 12,160                    |
| Orthologous gene pairs 2 | 12,756               | 12,756                  | 12,756                    |
| Orthologous gene pairs 3 | 10,923               | 10,923                  | 10,923                    |

Note: Statistic of genome assembly and annotation are based on Feng et al. (2020) for *P. huaijiensis* and Xiao et al. (2015) for *D. hygrometricum* unless noted. Orthologous gene pairs 1, *S. rexii* versus *P. huaijiensis*; 2, *S. rexii* versus *D. hygrometricum*; 3, *P. huaijiensis* versus *D. hygrometricum*.

<sup>a</sup>Based on Zhao et al. (2014).
<sup>b</sup>Based on Zhao et al. (2014).
<sup>c</sup>Num. of annotated gene models from genome in Xiao et al. (2015).
<sup>d</sup>Num. of predicted gene models from transcriptome in Xiao et al. (2015).
The distribution of Ks values for orthologous gene pairs between the species showed a peak between *S. rexii* and *P. huaijiensis* of /C24 0.2 (Figure 2); and peaks between *S. rexii* and *D. hygrometricum* of /C24 0.25; *P. huaijiensis* and *D. hygrometricum* of /C24 0.2 (Figure S4).

For *S. rexii* and *P. huaijiensis*, the difference of Ks distribution plots between paranomes and orthologous pairs are shown in detail in Figure 2. The Ks distribution plot of orthologous gene pair between *S. rexii* and *P. huaijiensis* and those separate paranomes for the two species were similar in shape, but the peaks for individual species were slightly shifted to lower values (Figure 2). The BGMM analyses of these species’ Ks plots indicated a small peak at Ks < 0.0625 with n > 3 (Figure S3). This small peak might be caused by tandem duplications rather than WGD.

### 3.5 * S. rexii* chloroplast and mitochondrial genome assembly

The chloroplast and mitochondrial genomes of *S. rexii* were assembled from the contigs in the draft genome assembly. In this assembly, six contigs were highly homologous to chloroplast sequences, and three to mitochondrial sequences (Figure 3; Table S9). The chloroplast contigs showed the highest average coverage of 2,694×, and the mitochondrial contigs 213× (Table 4, Table S9). Often, the same contigs were detected by both chloroplast and mitochondrial query searches, such as contig “2179_0,” but the bitscore and the contig coverage were distinguishable (Figure 3; Table S9). Based on the selected contigs 24,438 chloroplast raw reads and 2,462 mitochondrial raw reads were extracted for re-assembly of those organelle genomes.

The chloroplast reads were assembled into a circular genome of 152,571 bp, whereas the mitochondrial reads did not produce a circularized assembly and instead are represented by a large linear contig of 599,262 bp (Figure 3). The *S. rexii* chloroplast genome length and gene order were very similar to those of *D. hygrometricum* and *P. huaijiensis* as well as *A. thaliana* (Figures S5 and S6), although the mitochondrial gene order was highly divergent between *S. rexii*, *D. hygrometricum*, and *A. thaliana* (Figures S7 and S8), and the mitochondrial sequences could not be aligned between species (Figure S8). However, we confirmed that the original mitochondrial contigs aligned well with the final linearised *S. rexii* mitochondrial genome assembly (Figure S9).

### 3.6 Benchmark testing of the chloroplast and mitochondrial genome assembly pipeline: PLCL pipeline

The newly developed method for chloroplast and mitochondrial genome assembly used for *S. rexii* was based on an initial long-read assembly of ONT data and identified reads belonging to the nuclear, chloroplast, and mitochondrial genomes from read coverage differences. This method was further developed into the PLCL pipeline and tested with available ONT datasets of the model species *A. thaliana* and *O. sativa* subsp. *indica* (Figure 4). We initially tried to map *A. thaliana* KBS-Mac-74 ONT reads to the published hybrid genome
**Figure 3** Streptocarpus rexii chloroplast and mitochondrial genome assemblies. (a and b) Plots of blastn bitscores with Arabidopsis thaliana chloroplast or mitochondrion queries to the assembled genome contigs of S. rexii (x-axis), and the contig coverage (y-axis). The ID of contigs is shown for contigs selected as chloroplast or mitochondrial contigs. Green and red shaded markers indicate the selected chloroplast and mitochondrial contigs, respectively, found among the S. rexii genome contigs. (c) S. rexii circularized chloroplast genome assembly. (d) S. rexii mitochondrial genome assembly.
assembly of A. thaliana KBS-Mac-74 (NCBI GCA_900303355), but they did not map well, and we were unable to extract plastid contigs and reads (data not shown). Thus, we constructed an assembly solely with ONT reads using Wtdbg2, and obtained 353 contigs with an N50 of 9.8 Mbp and L50 of 5, where N50 is defined as the length of contigs representing half of the bases of the full assembly, and L50 the number of N50 contigs counted from the longest to the shortest (https://www.ncbi.nlm.nih.gov/assembly/help/). In blastn searches of those contigs with the chloroplast sequences as query, 26 contigs were hit, and with the mitochondrial sequence 31 (Figure S11). Several contigs were co-hit by chloroplast and mitochondrial queries, but their blastn bitscores were very different and distinguished them. K-means analyses were also used for a quick evaluation of coverage clustering. For the chloroplast, automatic cluster number selection ($k = 0$ in Algorithm::Kmeans) worked well, and four contigs were identified as chloroplast contigs. For the mitochondrion, $k = 0$ resulted in two clusters and the contigs in the c0 cluster (“ctg224,” “ctg70, Figure S11c) were also listed as chloroplast contigs (Figure S11a). However, “ctg224” and “ctg70” showed higher blastn bitscores to chloroplast than to mitochondria, and thus these were selected as chloroplast contigs. $K = 4$ seemed to distinguish the mitochondrial contigs (c1 and c2) from nuclear (c0) and chloroplast (c3) contigs. Contig “ctg31” was clustered as c2 by coverage, but it showed a low bitscore to mitochondrion query searches, and thus only “ctg55” and “ctgt36” were selected as the mitochondrial contigs (Figure S11).

In A. thaliana KBS-Mac-74, the 12,621 chloroplast reads and 8,239 mitochondrial reads extracted had an estimated coverage of 2,254× and 98×, respectively (Table 4). The chloroplast genome was successfully assembled from the extracted reads using Canu with the parameter sets ps2 and ps3. The resulting chloroplast contigs of A. thaliana KBS-Mac-74 were >190,000 and 148,000 bp when circularized (Table S11). Because the A. thaliana KBS-Mac-74 chloroplast sequence has not yet been reported, it was compared with the reference chloroplast sequence of A. thaliana Col-0 (154,470 bp). The A. thaliana Col-0 chloroplast was longer than the A. thaliana KBS-Mac-74 chloroplast in length, and the differences between the two genomes mostly concerned deletions in mononucleotide simple repeat regions. Although it is unknown whether this reflects differences between the lineages or is a result of assembly errors. The

| TABLE 4 Benchmark statistics of the plant contig clustering-based genome assembly (PLCL) pipeline |
|---------------------------------------------------------------|
| **Taxon** | **Streptocarpus rexii** | **Arabidopsis thaliana KBS-Mac-74** | **Oryza sativa subsp. indica IR64** |
| Num. total reads | 32,242,708 | 300,071 | 1,449,788 |
| Num. total bases (bp) | 158,689,714,464 | 3,421,779,258 | 9,275,443,298 |
| Num. total assembled contigs | 5,964 | 353 | 1,341 |
| Num. contigs | 6 | 3 | 4 |
| Average coverage | 2,694 | 213 | 2,255 |
| Num. reads | 24,438 | 2,462 | 12,621 |
| Read N50 (bp) | 21,036 | 20,894 | 19,778 |
| Assembly result | Whole cp/mt genome | Yes | Unknown |
|                  | Circular genome | Yes | No |

Note: Statistics for chloroplast (cp) and mitochondrial (mt) contigs and reads from the genome assemblies.

**FIGURE 4** Overview of the plant contig clustering-based genome assembly (PLCL) pipeline developed and applied in the present study, with steps, workflow, and programs embedded in the pipeline. cp, chloroplast; mt, mitochondrial
assembly quality was shown to be sensitive to the assembler and its parameter settings (Tables S10 and S11; Figure S12). With the Canu parameter setting ps1, the inverted repeat region was not well resolved and was inverted in the final sequence (see Figure S12a,d) but was properly assembled with settings ps2 and ps3 (Figure S12b–d). Ps3 resulted in the best assembly within the tested parameter settings and had fewer deletions/gaps when compared with the A. thaliana Col-0 chloroplast genome. Mitochondrial assemblies produced with Canu and Wtdbg2 did not assemble well and the resulting contigs had poorer contiguity (Figure S12f,g) than the initial two original mitochondrial contigs (Figure S12e). When the PLCL pipeline was tested with the O. sativa subsp. indica IR64 ONT dataset, the chloroplast genome was assembled well but not the mitochondrial genome (Table S11; Figure S12h,i), similar to the A. thaliana KBS-Mac74 ONT dataset.

4 | DISCUSSION

4.1 | A first nuclear genome assembly for the genus Streptocarpus

A nuclear genome assembly was successfully generated using two cells of ONT PromethION for S. rexii. This was remarkably cost effective compared with other methods, with an estimated sequencing cost of around $3,000 (https://medicine.yale.edu/keck/yca/services/ontprices/) for the 1-Gb haploid 1 C genome of S. rexii (Möller, 2018). This shows the dramatic decline in genome sequencing costs compared with earlier genome sequencing efforts. For example, the A. thaliana genome assembly cost around $100 million (reviewed in Li & Harkess, 2018).

Although the assembly is not yet refined to chromosome level, the draft genome is highly contiguous and will be a useful resource for genome-wide studies in S. rexii and the genus Streptocarpus, as well as in the family Gesneriaceae. The current S. rexii genome assembly has an N50 of 3.7 Mb, and therefore the contigs are much longer than the >1 Mbp threshold recommended for chromosome level scaffolding (Jung et al., 2020). This could be done with a range of technologies such as BioNano optical mapping (Deschamps et al., 2018) or Hi–C chromosome conformation capture (Dudchenko et al., 2017).

The assembled nuclear genome size was 766 Mb, which is smaller than the genome size predicted by flow cytometry of 929 Mb (Möller, 2018). It is known that it is difficult to assemble repetitive elements, where two identical reads belonging to different chromosomal positions are indistinguishable by the assembly programs (Tørresen et al., 2019). These unresolved repeats might be a reason for the smaller assembly size in this study, though further investigations are required here.

To achieve this highly contiguous ONT assembly, it was paramount to obtain high-quality high-molecular weight DNA (see also Mantere et al., 2019). Long read sequencing is known to be very sensitive to any DNA damage caused by drying the leaf tissue in silica gel or high-temperature treatments (e.g., >60°C), as well as the presence of any secondary metabolites such as polysaccharides, polyphenols, phensols, or chemical residues such as guanidinium isothiocyanate, EDTA, or ethanol (https://dnatech.genomecenter.ucdavis.edu/nanopore-sequencing-ont-promethion/). The method developed in this study was a modified nuclear isolation protocol that greatly reduced cytosol components prior to cell lysis, and used proteinase K cell lysis at 50°C to avoid the more conventional high temperature lysis at 60–65°C. Nuclear isolation-based methods seemed to be useful options for obtaining plant DNA suitable for long read sequencing (see also Zerpa-Catanho et al., 2021). We used Qiagen Genomic tip columns, because they were designed to retain 50–150-kb DNA fragments, fitting the criteria for ONT long read sequencing. Other more traditional protocols, such as CTAB or the commonly used Qiagen DNeasy kit, tested by us, did not provide sufficient DNA quality or quantity for S. rexii. In this plant, polysaccharides appear to represent the main obstacle, and the protocol developed here could well be applicable for other such problematic plants.

4.2 | Gesneriaceae annotation resources

A Maker annotation pipeline was employed to annotate the S. rexii genome. To have full function of the pipeline, a vast amount of lineage-specific annotation resources such as transcriptomes and repeat libraries are required (Holt & Yandell, 2011; https://gist.github.com/darenocard/bb1001ac1532dd4225b030cf0d1ce2), and these were generated in the present study from existing Gesneriaceae resources. Maker can utilize multi-species resources and for our purpose the genetic resources of OW Gesneriaceae available in public archives proved very useful. Moreover, all the genome repeat libraries of OW Gesneriaceae generated here have been deposited in public archives for general use. With these Gesneriaceae annotation resources, the Maker pipeline successfully annotated not only the S. rexii genome (with 45,045 genes) but also those of the other OW Gesneriaceae, D. hygrometricum and P. huaijiensis. In their original publications, 23,250 genes were annotated for D. hygrometricum (Xiao et al., 2015) and 31,328 for P. huaijiensis (Feng et al., 2020), whereas in this study, the number of genes annotated for D. hygrometricum (24,585 genes) and P. huaijiensis (42,685 genes) was slightly higher, indicating the strength and efficiency of the pipeline (Table 3). This genome annotation pipeline is likely to prove useful for further genomic characterization of the Gesneriaceae.

It is noteworthy that only 55%–60% of genes (24,585) could be annotated in D. hygrometricum compared with those annotated in S. rexii and P. huaijiensis. This might have been due to the relatively low contiguity of the D. hygrometricum genome assembly, because the gene models suggested from the transcriptomes was 49,374 genes, a similar number to those in S. rexii and P. huaijiensis. To annotate the remaining ~47% of genes in the D. hygrometricum genome would require an improved genome assembly.

The haploid genome size for D. hygrometricum was predicted to be 1,691 Mb by Xiao et al. (2015), which is significantly larger than genome size estimates for the two other study species here: 929 Mb.
for S. rexii (Müller, 2018) and 511 Mb for P. huajiensis (Feng et al., 2020). In these species, the haploid chromosome number is \( n = 9 \) (D. hygrometricum), \( n = 18 \) (P. huajiensis), and \( n = 16 \) (S. rexii) (see Table 3). The percentage of genome repeats was also the highest in D. hygrometricum (75.16%), lowest in P. huajiensis (54.10%), and with S. rexii falling towards the higher end (70.95%). Thus, there appears to be a trend that larger genomes in Gesneriaceae retain more genome repeats, with this showing an almost linear relationship to genome size. Long terminal repeat (LTR) retrotransposons are the most genome repeats, with this showing an almost linear relationship.

Assembly (Wang et al., 2018). However, the authors (Wang et al., 2020) reported that it did not assemble the entire chloroplast genome into a single contig, and it required manual curation. Organelle_PBA (Soomi et al., 2017) is a long read sequence assembler for chloroplast and mitochondrial genomes. It also selects the chloroplast or mitochondrial reads at the first step against a reference sequence, and the reads are then assembled. It requires scaffolding of assembled contigs using SSPACE-LongRead (Boetzer & Pirovano, 2014) in the final step. Our method, on the other hand, was able to assemble the chloroplast into a single contig at the assembly step. The different coverage observed when raw reads were remapped to the S. rexii genome assembly likely reflects the chloroplast and mitochondrion copy number in the sequencing libraries. For instance, two nuclear genomes exist in a diploid cell, but each chloroplast contains multiple genome copies and each cell contains several chloroplasts, and it was estimated that \( \sim 1,200 \) copies of the chloroplast genome are contained per mesophyll cell in A. thaliana (Sakamoto & Takami, 2018). The mitochondrial genome has a much more complex nature. It varies in structure and can be linear or circular, and often only a subset of mitochondrial genomes are kept in a cell depending on the cell condition in A. thaliana (Arimura, 2018). Unlike chloroplasts, mitochondrial genome copy number could be much smaller than the number of mitochondria themselves (Arimura, 2018), though the copy number per cell is still higher than for the nuclear genome. In an A. thaliana leaf for example, it was estimated that there may be 50 mitochondrial genome copies per cell (Cai et al., 2015). Thus, in a whole genome assembly, the contig coverage is expected to be “nuclear < mitochondria < chloroplast,” and this was demonstrated to be the case in this study. Because our method maps all reads to the whole genome, it has the advantage of eliminating cross-mapped reads between organelles.

The method applies simple blastn searches using a chloroplast or mitochondrial genome as the query sequence, and it was often the case that the same contigs were detected by both chloroplast and mitochondrion searches. However, these were distinguishable by differences in the contigs’ coverage and/or blastn bitscore values. It might also be an option to use an aligner such as minimap2 to map contigs to the query instead of blastn. The pipeline, which we call the PLCL pipeline, worked well for the chloroplast genome assembly of the plant species S. rexii, A. thaliana, and O. sativa, where perhaps the very high coverage helped.

For the mitochondrial genome assemblies, although the S. rexii ONT dataset (>158 Gbp) was assembled into one contig, the A. thaliana KBS-Mac-74 ONT dataset (MinION, 3 Gbp; Michael et al., 2018) and O. sativa subsp. indica IR64 ONT dataset (MinION, 9 Gbp; Tanaka et al., 2020) did not have sufficient coverage. The number of mitochondrial reads was higher in A. thaliana KBS-Mac-74 and O. sativa subsp. indica IR64 than in S. rexii, but the read length was longer in S. rexii (“Read N50” in Table 4), and thus the resulting coverage was higher in S. rexii. In addition, longer reads are known to improve the assembly’s contiguity. The pipeline was able to assemble the S. rexii mitochondrial genome into one linear scaffold (Table 4). The accuracy of the assembled S. rexii mitochondrial genome was supported as well assembled in series with its original mitochondrial genomes.
contigs (Figure S9). Mitochondrial genomes can exist as linear as well as circular forms (Arimura, 2018), and thus the S. rexii linear mitochondrial genome is likely correct and complete, rather than an incomplete assembly. It is difficult to judge the accuracy of the assembly of the mitochondrial genome from comparison with reference genomes, such as A. thaliana, because of the lack of conservation of gene order. This is well-known due to frequent mitochondrial fission and fusion events (Arimura, 2018; Kozik et al., 2019) that inhibits the alignment even between closely related genera such as Arabidopsis and Capsella (Figure S8d).

Thus, the PLCL pipeline appears to be coverage sensitive and requires high-volume input data. In addition, slight differences in the Canu parameter settings lead to differences in indels, and thus users are advised to test several assembler or parameter settings. The assembly, and this is perhaps true for any traditional long read assembly, might not be very accurate for detecting SNPs in mononucleotide repeat regions. In cases where researchers are interested in studying short repeats, it might be useful to polish the assembly with more accurate Illumina short reads. In addition, developments with more accurate long reads and improved algorithms for ONT base calling (https://nanoporetech.com/accuracy), as well as novel high fidelity PacBio HiFi methods, will hopefully greatly reduce sequence error rate in the future. Regardless of future developments, the PLCL pipeline has great potential as an automated chloroplast and mitochondrial genome assembler in projects where many large long read datasets are generated.

5 | CONCLUSIONS

In this study, we assembled the whole genome of S. rexii, including the chloroplast, mitochondrial, and nuclear genome. The estimated 929-Mb nuclear genome was assembled solely with the long read sequences into 5,855 scaffolds covering 766 Mb (83%). This provides a valuable example of a cost effective approach to generate a complete genome for a non-model organism. It is now possible to carry out genome-wide studies in Streptocarpus to pin down genes regulating its unique meristems. In addition, the resources and methods developed in this study were shown to be more widely applicable. The annotation resources are immediately useful for the genome annotation of Old World Gesneriaceae. The PLCL pipeline provides an effective chloroplast and mitochondrial genome assembly method with wider applications in plants. The DNA extraction method developed here is also likely to prove useful for other polysaccharide-rich plant species. This study provides not only a new genome but also shows the potential of long read sequencing methods to the wider scientific community.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The Streptocarpus rexii genome assembly and raw reads have been deposited in the European Nucleotide Archive (ENA; Study ID: PRJEB50530, Sample ID: ERS10392821, Genome assembly ID: GCA_929616715, Reads ID: ERR8482532, ERR8484618), and registered in the genome browser of Plant GARDEN (https://plantgarden.jp/en/list/t121488). The transcriptomes are also deposited in ENA (Streptocarpus ionanthus: ERZ5128686, Haberea rhodopensis: ERZ5129963). The genome repeat libraries (Data S1) have been deposited in Dfam (https://dfam.org/home) and will be available in a future release. The high-molecular weight (HMW) DNA extraction protocol and PLCL pipeline are deposited in protocol.io (HMW DNA extraction: dx.doi.org/10.17504/protocols.io.bempj5n, PLCL pipeline: dx.doi.org/10.17504/protocols.io.bx5dpq26).

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