De novo genome assembly of the land snail Candidula unifasciata (Mollusca: Gastropoda)

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Abstract

Among all molluscs, land snails are a scientifically and economically interesting group comprising edible species, alien species and agricultural pests. Yet, despite their high diversity, the number of genome drafts publicly available is still scarce. Here, we present the draft genome assembly of the land snail Candidula unifasciata, a widely distributed species along central Europe, belonging to the Geomitridae family, a highly diversified taxon in the Western-Palearctic region. We performed whole genome sequencing, assembly and annotation of an adult specimen based on PacBio and Oxford Nanopore long read sequences as well as Illumina data. A genome draft of about 1.29 Gb was generated with a N50 length of 246 kb. More than 60% of the assembled genome was identified as repetitive elements. In total, 22,464 protein-coding genes were identified in the genome, of which 62.27% were functionally annotated. This is the first assembled and annotated genome of the land snail Geomitridae and will serve as reference for future evolutionary, genomic and population genetic studies of this important and interesting group.

Keywords: annotation; de novo assembly; Geomitridae; land snails; long reads; molluscs; repeats

Introduction

Gastropods are the largest group among molluscs, representing almost 80% of the species in this second largest phylum. Within gastropods, snails occupy arguably the widest range of habitats of all metazoan taxa, ranging from deep sea vents to the alpine region and from deserts to polar regions. Land snail diversity is estimated around to 35,000 species (Solem 1984). Due to their low dispersal abilities, land snails have been employed long since in many evolutionary and population genetics studies (Cain and Sheppard 1954; Cameron 1992; Pfenninger et al. 1996; Chueca et al. 2017; Haponski et al. 2017). While these studies are mainly based on few loci, transcriptomes or mitochondrial genomes (Kang et al. 2016; Razkin et al. 2016; Romero et al. 2016; Korábek et al. 2019), only three whole nuclear genomes of land snails species are available so far, Achatina fulica (Guo et al. 2019), Achatina immaculata (Liu et al. 2021) and Cepaea nemoralis (Saenko et al. 2021).

The Geomitridae are one of the most diverse families of molluscs in the Western-Palearctic region. The family is composed of small to medium-sized species, characterized by presenting several (reproductive) adaptations to xeric habitats (Giusti and Manganelli 1987). Candidula unifasciata (NCBI: txid100452) is a small land snail species (5–10 mm) widely distributed along western Europe, from southern France and Italy to central and northern Europe (Figure 1). C. unifasciata inhabits dry meadows and open lowlands with rocks, being also present in gardens and vineyards. A recent molecular revision of Candidula (Chueca et al. 2018) revealed the polyphyly of the genus, and split the species that composed it into six genera, questioning the traditional anatomical classification. Previous work focused on the speciation (Pfenninger and Magnin 2001; Pfenninger et al. 2003) and climate-driven evolution (Pfenninger and Posada 2002; Pfenninger et al. 2007) of the genus, however, in depth analyses were hampered by the lack of genomic markers. Although, there are many taxonomical, phyleogeographical, and evolutionary studies concerning Geomitridae species (Pfenninger and Magnin 2001; Sauer and Hausdorf 2010; Brozzo et al. 2020), the lack of reference genomes makes it difficult to investigate deeper biological and evolutionary questions about geomitrids and other land snails species. Here, we present the annotated draft genome of C. unifasciata that will be a valuable resource for future genomic research of this important taxonomic group (Chueca et al. 2021).

Materials and methods

Sample collection, library construction, and sequencing

Several living individuals of C. unifasciata were collected from Winterscheid, Gilserberg, Germany (50.93° N, 9.04° E). Genomic DNA was extracted independently from two specimens using the phenol/chloroform method and quality was checked by gel...
electrophoresis and NanoDrop ND-1000 spectrophotometer (LabTech, USA). Genomic DNA from the first specimen (size distribution = 20 kb) was employed for Illumina and PacBio sequencing. A total of 5.6 μg of DNA was sent to Novogene (UK) for library preparation and sequencing. Then, a 300 base pair (bp) insert DNA libraries was generated using NEBNext® DNA Library Prep Kit and sequenced on 3 lanes of Illumina NovaSeq 6000 platform (150 bp paired-end [PE] reads). Quality of raw Illumina sequences was checked with FastQC (Andrews 2010). Low quality bases and adapter sequences were subsequently trimmed by Trimomatic v0.39 (Bolger et al. 2014). For PacBio sequencing, a DNA library was prepared from 5 μg of DNA using the SMRTbell template prep kit v1.0. Sequencing was carried out on 10 single-molecule real-time sequencing (SMRT) cells on an RSI instrument using P6-C4 chemistry.

To obtain Oxford Nanopore Technologies (ONT) long reads, we ran two flow cells on a MinION portable sequencer. Total genomic DNA from a second specimen (size distribution = 30 kb) was used for library preparation with the Ligation Sequencing kit (SQK-LSK109) from ONT, using the manufacturer’s protocols. Base calling of the reads from the two MinION flow cells was performed with guppy v4.0.11 (https://github.com/nanoporetech/pyguppyclient, last accessed on 07-06-2021), under default settings. Afterwards, ONT reads quality was checked with Nanoplot v1.28.1 (https://github.com/wdecoster/NanoPlot, last accessed on 07-06-2021) and reads shorter than 1000 bases and mean quality below seven were discarded by running Nanofilt v2.6.0 (https://github.com/wdecoster/nanofilt, last accessed on 07-06-2021) and reads shorter than 1000 bases and mean quality below seven were discarded by running Nanofilt v2.6.0 (https://github.com/wdecoster/nanofilt, last accessed on 07-06-2021).

Two living specimens, one adult and one juvenile, were kept in captivity for 3 days to purge the gut. After being purged, the whole bodies of both specimens were homogenized together into small pieces using steel balls and a Retsch Mill. Then, RNA was extracted following a standard Trizol extraction. The integrity of total RNA extracted was assessed on an Agilent 4200 TapeStation (Agilent, USA), after which, approximately 1 of total RNA extracted was assessed on an Agilent 4200 extract 0.7 quality. Nano RNA was isolated from the gut tissue of both specimens using propidium iodide-stained nuclei described in (Hare and Johnston 2012). Foot tissue of one fresh adult sample of C. unifasciata and neural tissue of the internal reference standard Acheta domesticus (female, 1 C = 2 Gb) mixed and chopped with a razor blade in a petri dish containing 2 ml of ice-cold Galbraith buffer. The suspension was filtered through a 42-μm nylon mesh and stained with the intercalating fluorochrome propidium iodide (PI, Thermo Fisher Scientific) and treated with RNase II A (Sigma-Aldrich), each with a final concentration of 25 μg/ml. The mean red PI fluorescence signal of stained nuclei was quantified using a Beckman-Coulter CytoFLEX flow cytometer with a solid-state laser emitting at 488 nm. Fluorescence intensities of 5000 nuclei per sample were recorded. We used the software CytExpert 2.3 for histogram analyses. The total quantity of DNA in the sample was calculated as the ratio of the mean red fluorescence signal of the 2 C peak of the stained nuclei of the C. unifasciata sample divided by the mean fluorescence signal of the 2 C peak of the reference standard times the 1 C amount of DNA in the standard reference. Four replicates were measured to minimize possible random instrumental errors. Furthermore, we estimated the genome size by coverage from mapping reads used for genome assembly back to the assembly itself using backmap v0.3 (https://github.com/schellt/backmap; Schell et al. 2017, last accessed on 07-06-2021). In brief, the method divides the number of mapped nucleotides by the mode of the coverage distribution. By doing so, the length of collapsed regions with many fold increased coverage is taken into account.

**Genome assembly workflow**

Several *de novo* genome assemblies were tested under different methods (see Supplementary Table S1). The pipeline, which showed the best genome, was selected to continue further analyses. The draft genome was constructed from PacBio long reads using wtdbg2 v2.5 (Ruan and Li 2020), followed by three polishing rounds of Racon 1.4.3 (Vaser et al. 2017) and three polishing rounds of Pilon 1.23 (Walker et al. 2014). After that, Illumina and PacBio reads were aligned to the assembly using backmap.pl v0.3 to evaluate coverage distribution. Then, Purge Haplotigs (Roach et al. 2018) was employed, under default parameters and cutoff values of 15, 72, and 160 to identify and remove redundant contigs.

**Scaffolding and gap closing**

To further improve the assembly, we applied two rounds of scaffolding and gap closing to the selected genome assembly. The
genome was first scaffolded with the PacBio and ONT reads by LINKS v1.8.7 (Warren et al. 2015) and then with RNA reads by Rascaf v1.0.2 (Song et al. 2016). After each scaffolding step LongRead Gapcloser v1.0 (Xu et al. 2019) was run once, followed by three rounds of polishing former gap regions using Racon v1.4.3. BlobTools v1.0 (Kumar et al. 2013; Laetsch and Blaxter 2017) was employed to screen genome assembly for potential contamination by evaluating coverage, GC content and sequence similarity against the NCBInr database of each sequence. The resulting assembly was compared in terms of contiguity using Quast v5.0.2 (Gurevich et al. 2013), and evaluated for completeness by BUSCO v3.0.2 (Simão et al. 2015) against metazoa_odb9 data set.

Transcriptome assembly
RNA reads were checked for quality and trimmed, as was explained above. The transcriptome was assembled using Trinity v2.9.1 (Haas et al. 2013). Then, the transcriptome assembly was evaluated for completeness by BUSCO v3.0.2 against the metazoa_odb9 data set. Moreover, the clean RNA-seq reads from different specimens were aligned against the draft assembly by HISAT2 v2.1.0 (Kim et al. 2015).

Repeat annotation
RepeatModeler v2.0 (Smir and Hubley 2008) was run to construct a de novo repeat library from the genome assembly. The resulting repeat library was employed by RepeatMasker v4.1.0 (http://www.repeatmasker.org/, last accessed on 07-06-2021) to annotate and mask the genome assembly.

Gene prediction and functional annotation
Genes were predicted by using different methods. First, genes models were predicted ab initio based on SNAP v. 2006-07-28 (Korf 2004) and the candidate coding regions within the assembled transcript were identified with TransDecoder v5.5.0 (https://github.com/TransDecoder/, last accessed on 07-06-2021). Second, we used homology-based gene predictions by aligning protein sequences from SwissProt (2020-04) to the C. unifasciata masked genome with EXONERATE v2.2.0 (Slater and Birney 2005) and by running GeMoMa v1.7.1 (Keilwagen et al. 2016, 2018) taking five gastropod species as reference organisms. The selected species were Pomacea canaliculata [GCF_003073045.1; (Liu et al. 2019)], Aplysia californica [GCF_000002075.1; Elysia chlorotica [GCA_003991915.1; (Cai et al. 2019)], Radix auricularia [GCA_002072015.1; (Schell et al. 2017)], Chrysomallon squamiferum [GCA_012295275.1; (Sun et al. 2020)]. First, from the mapped RNA-seq reads, introns were extracted and filtered by the GeMoMa modules ERE and Denoiseltron. Then, we ran independently the module GeMoMa pipeline for each reference species using mmseqs2 v2.8.7875 (Steinegger and Söding 2017, 2018) and including the mapping of species own RNA-seq data. The five gene annotations were then combined into a final annotation file by using the GeMoMa modules GAF and AnnotationFinalizer. Finally, we aligned C. unifasciata transcripts against the masked genome using PASA v2.4.1 (Campbell et al. 2006) as implemented in autoAug.pl from Augustus v3.3.3 (Stanke et al. 2004).

Gene prediction data from each method were combined using EvidenceMolder v1.1.1 (Haas et al. 2008) to obtain a consensus gene set for the C. unifasciata genome. Gene models from GeMoMa and SNAP were converted to EVM compatible gff3 files using EVM’s included scripts and combined with CDS identified by TransDecoder into a gene predictions file. After that, EVM was run including gene model predictions, protein and transcript alignments and repeat regions to produce a reliable consensus gene set (Supplementary Table S2).

Predicted genes were functionally annotated by BLAST search against the Swiss-Prot database with an e-value cutoff of 10⁻⁶. InterProScan v5.39.77 (Quevillon et al. 2005) was used to predict motifs and domains, as well as Gene ontology (GO) terms.

Data availability
All raw data generated for this study (Illumina, PacBio, MiniION, and RNA-seq reads) are available at the European Nucleotide Archive database (ENA) under the Project number: PRJEB41346. The final genome assembly and annotation can be found under the accession number GCA_905116865.

The execution of this work involved using many software tools which are listed in Table 1. Supplementary File S1 contains BUSCO results. Supplementary File S2 contains de novo library of repeats generated by RepeatModeler. Supplementary File S3 contains C. unifasciata repeats. Supplementary File S4 contains sequences of protein-coding genes predicted in the C. unifasciata genome. Supplementary File S5 contains proteins predicted in the C. unifasciata genome. Supplementary File S6 contains genome annotation. Supplementary Table S1 contains a comparison between draft genomes assemblies for C. unifasciata obtained by different tools. Supplementary Table S2 contains weights values of evidences for EVM analysis. Supplementary Table S3 contains RepeatMasker results. Supplementary Material is available at figshare: https://doi.org/10.25387/g3.14237828.

Results and discussion

Genome assembly
The calculated DNA content through flow cytometry experiments was 1.54 Gb. The genome size estimation by back-mapped Illumina read coverage resulted in 1.42 Gb. The estimated heterozygosity by GenomeScope of the specimen employed for genome assembly, based on 21-mers, was around 1.09% (Figure 2A), being in the range of other land snail genomes (Guo et al. 2019; Saenko et al. 2021). We generated sequence data for a total coverage of approximately 120.6X (185.73 Gb), 25.6X (39.43 Gb, N50: 9.9 kb), and 2.1X (3.24 Gb, N50: 16.2 kb) of Illumina, PacBio, and ONT reads respectively. Although we employed HMW-DNA for the ONT sequencing, pores collapsed and started dying very quick, generating less amount of data than expected. After scaffolding with long reads (PacBio and ONT) and RNA data, we produced a draft genome assembly of 1.29 Gb with 8586 scaffolds and a scaffold N50 of 246 kb (Table 2). Contiguity value was also good, with a scaffold NG50 of 341 kb, similar to C. nemoralis genome assembly. Completeness evaluation by BUSCO against the metazoa_odb9 data set showed high values, recovering more than the 92% as complete and less than the 6% as missing genes for both, assembly and annotation, analyses (Table 2, Supplementary File S1). These results were in the range of other recent high quality gastropod genome assemblies (Schell et al. 2017; Liu et al. 2018; Guo et al. 2019; Sun et al. 2020), being slightly better than closest relative assembly of C. nemoralis (Saenko et al. 2021). For genome quality evaluation, we compared the C. unifasciata draft genome generated with other mollusc genomes publicly available. This comparison showed high quality in terms of contig number and scaffold N50 among land snail genomes. The mapping of the Illumina reads against the final genome assembly showed a mapping rate of 98.56%, as well as a removal of most redundant regions (Figure 2B). Finally, BlobTools analysis shows a small fraction of the assembly assigned to phyla other than Mollusca.
(e.g., Arthropoda, Chordata among others). This is probably due to incompleteness of the nt database—where the taxonomic assignment is based on—and resulting random hits to more or less conserved sequences throughout the different phyla. Therefore, focusing on GC and coverage distribution, no contamination could be identified (Figure 3), indicating the reliability of the data.

**Genome annotation**

We estimated the total repeat content of the *C. unifasciata* genome assembly around 61.10% (Table 3, Supplementary Table S3, Supplementary Files S2 and S3), values slightly smaller than other land snails genomes (Guo et al. 2019; Saenko et al. 2021). Approximately one third of the assembled genome (33.96%) was identified as Transposable elements (TEs) such as long interspersed nuclear elements (LINEs; 25.03%), short interspersed nuclear elements (SINEs; 4.23%), long tandem repeats (LTR; 0.60%), and DNA transposons (4.10%).

We predicted 22,464 protein coding genes in the *C. unifasciata* genome (Table 4, Supplementary Files S4 and S5) by using a homology-based gene prediction and EVM (Supplementary File S6). Among the identified proteins, 13,221 (62.27%) could be annotated with at least one GO term. Finally, 21,231 proteins (94.51%)
**Figure 3** Blob plot showing read coverage, GC content and size of each scaffold. Size of the blobs correspond to size of the scaffold and color corresponds to taxonomic assignment based on a blast search against the nt database.

**Table 3** Repeat statistics

| Assembly       | LINE   | SINE   | LTR    | DNA    | Unclassified | SmRNA   | Others     | Total (%) |
|----------------|--------|--------|--------|--------|--------------|---------|------------|-----------|
| *C. unifasciata* | 1,253,318 | 427,509 | 11,975 | 298,828 | 1,334,718    | 413,197 | 708,740    | 61.1      |
| *C. nemoralis*   | 2,820,864 | 342,120 | 209,476 | 443,363 | 4,400,828    | 444,489 | 1,267,814  | 76.4      |

*De novo* and homology based repeat annotations as reported by RepeatMasker and RepeatModeler for *C. unifasciata* and comparison with *C. nemoralis*. Families of repeats included here are long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs), long tandem repeats (LTR), DNA transposons (DNA), unclassified (unknown) repeat families, small RNA repeats (SmRNA), and others (consisting of small, but classified repeat groups). The last column represents the total percentage of base pairs annotated as repeats.
Table 4  Annotation statistics of the predicted protein-coding genes for C. unifasciata genome

|                      | C. unifasciata |
|----------------------|---------------|
| Number               |               |
| Gene                 | 22,464        |
| mRNA                | 22,464        |
| Exon                 | 147,783       |
| CDS                  | 147,783       |
| Mean                 |               |
| mRNAs/gene          | 1             |
| CDSs/mRNA            | 6.58          |
| Median length        |               |
| Gene                | 11,931        |
| mRNA                | 11,931        |
| Exon                 | 129           |
| Intron               | 2,025         |
| CDS                  | 129           |
| Total space (Mb)     |               |
| Gene                | 379,573,459   |
| CDS                  | 26,582,739    |
| Single               |               |
| CDS mRNA             | 3,562         |

Table 1  Software employed in this work, their package version and source availability. All url were last accessed on 07-06-2021.

| Name                  | Version | Url                                                                 |
|-----------------------|---------|----------------------------------------------------------------------|
| Flye                  | 2.6     | https://github.com/fenderglass/Flye                                   |
| wtdbg2                | 2.5     | https://github.com/ruanjue/wtdbg2                                     |
| Canu                  | 1.9     | https://github.com/marbl/canu                                         |
| Racon                 | 1.4.3   | https://github.com/isovic/racon                                        |
| Pilon                 | 1.23    | https://github.com/broadinstitute/pilon                               |
| Quast                 | 5.0.2   | https://github.com/ablalab/quast                                      |
| BUSCO                 | 3.0.2   | https://busco.ezlab.org/                                              |
| BlobTools             | 1.1.1   | https://github.com/DRL/blobtools                                      |
| LINKS                 | 1.8.7   | https://github.com/bcgsc/LINKS                                        |
| Rascaf                | 1.0.2   | https://github.com/mourisli/Rascaf                                    |
| Long-Read Gapcloser   | 1.0     | https://github.com/CAFS-bioinformatics/LR_Gapcloser                  |
| FastQC                | 0.11.9  | https://www.bioinformatics.babraham.ac.uk/projects/fastqc/           |
| Trimmomatic           | 0.39    | https://multiqc.info/                                                |
| MultiQC               | 1.9     | http://qb.cshl.edu/genomescope/                                      |
| GenomeScope           | 1.0     | http://www.jstacs.de/index.php/GeMoMa                               |
| Trinity               | 2.9.1   | https://github.com/trinityrnaseq/trinityrnaseq/wiki                   |
| GeMoMa                | 1.6.4   | http://www.jstacs.de/index.php/GeMoMa                               |
| MMseqs2               | 5877873 | https://github.com/soeinglab/MMseqs2                                 |
| Augustus              | 3.3.3   | https://github.com/Gaius-Augustus/Augustus                           |
| TransDecoder          | 5.5.0   | https://github.com/TransDecoder                                       |
| SNAP                  | 2006-07-28 |                                                        |
| EXONERATE             | 2.2.0   | https://www.ebi.ac.uk/about/vertebrate-genomics/software/exonerate-manual |
| PASA                  | 2.4.1   | https://github.com/PASApipeline/PASApipeline                         |
| EVidenceMolder        | 1.1.1   | https://evidencemodeler.github.io                                   |
| guppy                 | 4.0.11  | https://github.com/nanoporetech/pyguppyclient                        |
| Nanoplot              | 1.28.1  | https://github.com/wdecost/NanoPlot                                  |
| Nanofilt              | 2.6.0   | https://github.com/wdecost/nanofilt                                  |
| backmap.pl            | 0.3     | https://github.com/schellf/backmap                                    |
| SAMtools              | 1.1.0   | https://github.com/samtools/samtools                                 |
| BWA                   | 0.7.17  | https://github.com/lh3/bwa                                            |
| minimap2              | 2.17    | https://github.com/lh3/minimap2                                     |
| Qualimap              | 2.2.1   | http://qualimap.conesalab.org/                                      |
| bedtools              | 2.28.0  | https://bedtools.readthedocs.io/en/latest/                           |
| Rscript               | 3.6.3   | https://www.r-project.org/                                           |
| RepeatModeler         | 2.0     | http://www.repeatmasker.org/RepeatModeler/                           |
| RepeatMasker          | 4.1.0   | http://www.repeatmasker.org/RepeatMasker/                            |
| HISAT2                | 2.1.0   | http://daehwankimlab.github.io/                                     |
were assigned to at least one of the databases used within InterProScan (Table 4). BUSCO and functional annotations results indicated high quality. Total protein-coding genes was in the range of other gastropods annotations (Schell et al. 2017; Liu et al. 2018; Guo et al. 2019), however this number represented only the half of its closest relative C. nemoralis (Saenko et al. 2021). Nevertheless, C. nemoralis genome annotation showed less completeness, with significant numbers of fragmented and missing BUSCO’s. These results could indicate that annotated genes are too fragmented and therefore, the real gene number of C. nemoralis annotation might be similar to other gastropods such as C. unifasciata.

Conclusions

Here, we present a high quality draft assembled and annotated genome of the land snail C. unifasciata. The obtained genome is comparable with other land snail and Gastropoda genomes publicly available. The new genome resource will be reference for further population genetics, evolutionary and genomic studies of this highly world-wide diverse group.

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Conflicts of interest

None declared.

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