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Finding Nemo 2.0: Hybrid assembly with Oxford Nanopore and Illumina reads dramatically improves the Clownfish (Amphiprion ocellaris) genome

--Manuscript Draft--

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Abstract:	Background: Some of the most widely recognised coral-reef fishes are clownfish or anemonefishes, members of the family Pomacentridae (subfamily: Amphiprioninae). They are popular aquarium species due to their bright colours, adaptability to captivity and fascinating behavior. Their breeding biology (sequential hermaphrodites) and symbiotic mutualism with sea anemones have attracted much scientific interest. Moreover there are some curious geographic-based phenotypes which warrant investigation. Leveraging on the advancement in Nanopore long read technology, we report the first hybrid assembly of the clown anemonefish (Amphiprion ocellaris) genome utilizing Illumina and Nanopore reads, further demonstrating the substantial impact of modest long read sequencing data sets on improving genome assembly statistics. Findings: We generated 43 Gb of short Illumina reads and 9 Gb of long Nanopore reads representing an approximate genome coverage of 54× and 11×, respectively, based on an estimated k-mer predicted genome size of 794 Mb. The final assembled genome size is contained in 6,404 scaffolds with an accumulated length of 880 Mb (96.3% BUSCO-calculated genome completeness). Compared to the Illumina-only assembly, the hybrid approach generated 93% less scaffolds with 18-fold increase in N50 length (401 kb) and increased the genome completeness by an additional 16%. A total of 27,240 high quality protein-coding genes were predicted from the clown anemonefish, 26,211 (96%) of which were annotated functionally with information from either sequence homology or protein signature searches. Conclusions: We present the first genome of any anemonefish and demonstrate the value of low coverage (~11×) long Nanopore reads sequencing in improving both genome contiguity and completeness. The near-complete genome of A. ocellaris will be an invaluable molecular resource for supporting a range of genetic, genomic and phylogenetic studies specifically for clownfish and more generally for other related fish species of the family Pomacentridae.
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Finding Nemo 2.0: Hybrid assembly with Oxford Nanopore and Illumina reads dramatically improves the Clownfish (*Amphiprion ocellaris*) genome

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Abstract

Background: Some of the most widely recognised coral-reef fishes are clownfish or anemonefishes, members of the family Pomacentridae (subfamily: Amphiprioninae). They are popular aquarium species due to their bright colours, adaptability to captivity and fascinating behavior. Their breeding biology (sequential hermaphrodites) and symbiotic mutualism with sea anemones have attracted much scientific interest. Moreover there are some curious geographic-based phenotypes which warrant investigation. Leveraging on the advancement in Nanopore long read technology, we report the first hybrid assembly of the clown anemonefish (*Amphiprion ocellaris*) genome utilizing Illumina and Nanopore reads, further demonstrating the substantial impact of modest long read sequencing data sets on improving genome assembly statistics.

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Conclusions: We present the first genome of any anemonefish and demonstrate the value of low coverage (~11×) long Nanopore reads sequencing in improving both genome contiguity and completeness. The near-complete genome of *A. ocellaris* will be an invaluable molecular resource for supporting a range of genetic, genomic and phylogenetic studies specifically for clownfish and more generally for other related fish species of the family Pomacentridae.

Keywords: clownfish, long reads, genome, transcriptome, hybrid assembly

Data description

The clown anemonefish, *Amphiprion ocellaris* (NCBI Taxon ID: 80972, Fish Base ID:6509), is a well-known tropical marine fish species among the non-scientific community especially following the Pixar film "Finding Nemo" and its sequel "Finding Dory" [1]. The visual appeal of *A. ocellaris* due to its bright coloration and behaviour and ease of husbandry has maintained a strong global demand for this species in the marine aquarium trade driving a fine balance between positive environmental awareness versus sustainable ornamental use [1, 2]. Further, given high survival rates and ability to complete their lifecycle in captivity, captive-breeding programs to partially sustain their global trade have been successful [3]. For the scientific community, *A. ocellaris* or anemonefishes in general, are actively studied due to their intriguing reproductive strategy i.e. sequential hermaphroditism [4-7] and mutualistic relationships with sea anemones [8-12]. Phenotypic body-colour variation based on host-anemone use and geography also pose additional questions regarding adaptive genetic variation [13]

In recent years, concurrent with the advent of long read sequencing technologies [14], several studies have explored combining short but accurate Illumina reads with long but less accurate Nanopore/PacBio reads to obtain genome assemblies that are usually more contiguous with higher completeness than assemblies based on Illumina-only reads [15-19]. To further contribute to the evaluation of long read technology in fish genomics [15], we sequenced the whole genome of *A. ocellaris* using Oxford Nanopore and Illumina technologies and demonstrate that hybrid assembly of long and short reads greatly improved the quality of genome assembly.

Whole genome sequencing

Tissues for genome assembly and as reference material were sourced from the collection of the Museum and Art Gallery of the Northern Territory (NTM). The samples used for DNA extraction and subsequent whole genome sequencing were from freshly-vouchered captive bred *A. ocellaris* specimens representing a unique black and white colour phenotype found only in the Darwin Harbour region, Australia (NTM A3764, A4496, A4497).

Genomic DNA was extracted from multiple fin clip and muscle samples using E.Z.N.A.® Tissue DNA Kit (Omega Bio-tek, Norcross, GA). For Illumina library

prep, approximately 1µg of gDNA from isolate A3764 was sheared to 300 bp using a Covaris Focused-ultrasonicator (Covaris, Woburn, MA) and subsequently processed using TruSeq DNA sample prep kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Paired-end sequencing was performed on a single lane of HiSeq 2000 (Illumina, San Diego, CA) located at the Malaysian Genomics Resource Centre Berhad. Two additional libraries were constructed from specimen NTM A3764 and both libraries were sequenced on the MiSeq (2×300 bp setting) located at the Monash University Malaysia Genomics Facility.

To generate Oxford Nanopore long reads, approximately 5 µg of gDNA was extracted from isolates NTM A4496 and A4497, size-selected (8 – 30 kb) with a BluePippin (Sage Science, Beverly, MA) and processed using the Ligation sequencing 1D kit (Oxford Nanopore, UK) according to the manufacturer's instructions. Three libraries were prepared and sequenced on three different R9.4 flowcells using the MinION portable DNA sequencer (Oxford Nanopore, UK) for 48 hours.

Sequence read processing

Raw Illumina short reads were adapter-trimmed with Trimmomatic v.0.36 (*ILLUMINACLIP:2:30:10*, *MINLEN:100*) [20] followed by a screening for vectors and contaminants, using Kraken v.0.10.5 [21] based on the MiniKraken DB. Kraken-unclassified reads i.e. non-microbial/viral origin were aligned to the complete mitogenome of NTM A3764 (See "Mitogenome Assembly") to exclude sequences of organellar origin. This results in a total of 42.35 Gb "clean" short reads. Nanopore reads were base-called from their raw FAST5 files using the Oxford Nanopore propriety base-caller, Albacore version 2.0.1. Applying a minimum and maximum length cut-off of 500 bp and 1Mbp, respectively, this study produced a total of 8.95 Gbp in 895,672 Nanopore reads (N_{50}: 12.7 kb)

Genome size estimation

K-mer counting with the filtered Illumina reads was performed with Jellyfish v.2.2.6 [22], generating k-mer frequency distributions of 17-, 21- and 25-mers. These histograms were processed by GenomeScope [23] that estimated a genome size of 791 to 794 Mb with approximately 80% of unique content and a heterozygosity level of 0.6% (Supplemental Figure 1). Given that we had previously excluded adapters as

well as sequences from contaminant or organellar sources, the max kmer coverage filter was not applied (*max kmer coverage: -1*). The genome size estimated from this approach is within the range of sizes listed for other *Amphiprion* species (792 Mb - 1.2 Gb) as reported on the Animal Genome Size Database (http://www.genomesize.com accessed on 11th November 2017)

Hybrid genome assembly

Both short-read-only and hybrid *de novo* assemblies were performed with MaSuRCA v.3.2.2 [24]. During hybrid assembly, errors were encountered in the fragment correction step of the Celera Assembler (CA). To overcome this, given that the CA assembler is no longer maintained, we disabled the *frgcorr* step based on one of the developer's recommendations and the hybrid assembly was subsequently improved with 10 iterations of Pilon v.1.22 [25], using short reads to correct bases, fix mis-assemblies and fill assembly gaps. To assess the completeness of the genome, BUSCO v.3.0.2 [26] was used to locate the presence or absence of the Actinopterygii-specific set of 4,584 single copy orthologs (OrthoDB v9).

The short-read-only and hybrid assemblies yielded total assembly sizes of 851 Mb and 880Mb, respectively. Inclusion of Nanopore long reads for hybrid assembly representing approximately 11× genome coverage led to a 94% decrease in the number of scaffolds (> 500 bp) from 106,526 to 6,404 scaffolds and an 18-fold increase in the scaffold N_{50} length from 21,802 bp to 401,715 bp (Table 1). In addition, the genome completeness was also substantially improved in the hybrid assembly, with BUSCO detecting complete sequences of 96.3% (4,417/4,584) of single-copy orthologs in the Actinopterygii-specific dataset.

Transcriptome sequencing and assembly

Total RNA extraction from RNAshield-preserved whole body and muscle tissues of isolate A4496 used Quick-RNA MicroPrep (Zymo Research Corpt, Irvine, CA) according to the manufacturer's protocols. After assessing total RNA intactness on the Tapestation2100 (Agilent), mRNA was enriched using NEBNext Poly(A) mRNA magnetic isolation kit (NEB, Ipwich, MA) and processed with NEBNext Ultra RNA library prep kit for Illumina (NEB, Ipwich, MA). Libraries from both whole body and muscle tissues were sequenced on a fraction of MiSeq V3 flowcell (1×150 bp). Single-end reads from both libraries in addition to two publicly available *A. ocellaris*

transcriptome sequencing data (SRR5253145 and SRR5253146, Bioproject ID: PRJNA374650) were individually assembled using Scallop v0.10.2 [27] based on HiSat2 [28] alignment of RNA-sequencing reads to the newly generated *A. ocellaris* genome. The transcriptome assemblies were subsequently merged using the tr2aacds pipeline from the EvidentialGene [29] package and similarly assessed for completeness using BUSCO version 3 [26]. The final non-redundant transcriptome assembly, which was subsequently used to annotate the *A. ocellaris* genome, contains 25,264 contigs/isotigs (putative transcripts) with an accumulated length of 68.4 Mb and BUSCO-calculated completeness of 92.8% (Table 1).

Genome annotation

Protein-coding genes were predicted with the MAKER v.2.31.9 genome annotation pipeline [30]. A total of three passes were run with MAKER2; the first pass was based on hints from the assembled transcripts as RNA-seq evidence (*est2genome*) and protein sequences from 11 fish species downloaded from Ensembl [31] (*protein2genome*), whereas the second and third passes included gene models trained from the first (and then, second) passes with *ab initio* gene predictors SNAP [32] and Augustus [33]. In the final set of genes predicted, sequences with Annotation Edit Distance (AED) values less than 0.5 were retained. A small AED value suggests a lesser degree of difference between the predicted protein and the evidences used in the prediction (i.e. fish proteins, transcripts). This resulted in a final set of 27,240 protein-coding genes with an average AED of 0.14 (Table 1). A BUSCO analysis on the completeness of the predicted protein dataset detected the presence of 4,259 (92.9%) single-copy orthologs from the Actinopterygii-specific dataset.

Further, to infer putative function of these predicted proteins, NCBI's *blastp* v.2.6.0 (*-evalue 1e-10, -seg yes, -soft_masking true, -lcase_masking*) [34] was used to find homology to existing vertebrate sequences in the non-redundant (NR) database. Applying a hit fraction filter to include only hits with ≥70 % target length fraction, the remaining un-annotated sequences were subsequently aligned to all sequences in the NR database. With this method, 20,107 proteins (74%) were annotated with a putative function based on homology. Additionally, InterProScan v.5.26.65 [35] was used to examine protein domains, signatures and motifs present in the predicted protein sequences. This analysis detected domains, signatures or motifs for 26,211 proteins (96%). Overall, 96% of the predicted clown fish protein-coding genes were

functionally annotated with information from at least one of the two approaches.

Mitogenome recovery via genome skimming

Genome skimming [36, 37] was performed on three additional *A. ocellaris* individuals from known locality (Supplemental Table 1). Mitogenome assembly was performed with MITObim version 1.9 [38] using the complete mitogenome of *A. ocellaris* (GenBank: NC009065.1) as the bait for read mapping. The assembled mitogenomes were subsequently annotated with MitoAnnotator [39]. Consistent with original broodstock collection from northern Australia, the captive bred black and white *A. ocellaris* NTM A3764 exhibits strikingly high whole mitogenome nucleotide identity (99.98%) to sample NTM A3708 as a wild collection from Darwin Harbour, Australia. In addition, the overall high pair-wise nucleotide identity (> 98%) of NTM A3764 to newly generated and publicly available *A. ocellaris* whole mitogenomes further supports its morphological identification as *A. ocellaris* (Supplemental Table 1).

Identification of the *cyp19a1a* gene associated with sexual differentiation

The validated *cyp19a1a* enzyme of *Danio rerio* (Uniprot: O42145) was used as the query (E-value = 1e-10) for blastp search against the predicted *A. ocellaris* proteins. The top blast hit, AMPOCE_00012675-RA (71.5% protein identity to O42145), was searched (tblastn) against the NCBI TSA database (Taxon: *Amphirion*) and showed strikingly high protein identity (99%) to a translated RNA transcript from *Amphiprion bicinctus* (c183337_g1_i2 : GDCV01327693) [5]. The *cyp19a1a* gene codes for a steroidogenic enzyme that converts androgens into estrogens [40] and was recently shown to be instrumental during sex change in *Amphiprion bicinctus* as evidenced by significant correlation and differential expression of this gene between male and mature females [5]. We also observed similar profile based on mapping of RNA reads from the publicly available male and female transcriptomes of *A. ocellaris* to the *cyp19a1a* gene region as visualized using Integrative Genomics Viewer [41] (Figure 2). The *A. ocellaris cyp19a1a* gene is located on a 419 kb scaffold and is spanned by multiple Minimap2-aligned Nanopore reads [42]. It is noteworthy that in the Illumina-only assembly, this gene is fragmented and located on 3 relatively short scaffolds (Figure 2).

Conclusion

We present the first clownfish genome and demonstrate the value of low coverage (~11×) Nanopore long read sequencing in improving both genome contiguity and completeness. The near-complete genome of *A. ocellaris* will be an invaluable molecular resource for supporting a range of genetic, genomic, and phylogenetic studies specifically for clownfish and more generally for other related fish species of the family Pomacentridae.

Availability of supporting data

Data supporting the results of this article will be available in the GigaDB repository. Raw Illumina and Nanopore reads generated in this study are available in the Sequence Read Archive (SRP123679) whereas Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession NXFZ00000000, both under BioProject PRJNA407816.

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Competing interests

The authors declare that they have no competing interests

Figure Legends

Figure 1. The clown anemonefish (*Amphiprion ocellaris*): Photo by Michael P. Hammer

Figure 2. Mapping of MinION long reads, Illumina-assembled scaffolds and RNA-sequencing reads of male and female *A. ocellaris* to genomic region containing the *cyp19a1a* gene. Transcripts per million (TPM) values were calculated using Kallisto version 0.43.1 [43].

Supplemental Figure 1. Genome profiling of *A. ocellaris* based on Illumina short reads

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Table 1. Genome and Transcriptome statistics of the clownfish (*Amphiprion ocellaris*) genome

	Illumina (≥500bp)	**Illumina + Nanopore (≥500bp)**
Genome Assembly		
Contig statistics		
Number of contigs	133,997	7,810
Total contig size (bp)	851,389,851	880,159,068
Contig N_{50} size (bp)	15,458	323,678
Longest contig (bp)	204,209	2,051,878
Scaffold statistics		
Number of scaffolds	106,526	6,404
Total scaffold size (bp)	852,602,726	880,704,246
Scaffold N_{50} size (bp)	21,802	401,715
Longest scaffold (bp)	227,111	3,111,502
GC / AT / N (%)	39.6 / 60.2 / 0.14	39.4 / 60.5 / 0.06
BUSCO Genome Completeness		
Complete	3,691 (80.5%)	4,417 (96.3%)
Complete and single copy	3,600 (78.5%)	4,269 (93.1%)
Complete and duplicated	91 (2.0%)	148 (3.2%)
Fragmented	534 (11.6%)	63 (1.4%)
Missing	359 (7.9%)	104 (2.3%)
Transcriptome Assembly		
Number of contigs	25,364	
Total length (bp)	68,405,796	
Contig N_{50} size (bp)	3,670	
BUSCO completeness		
Complete	4,253 (92.8%)	
Complete and single-copy	4,128 (90.1%)	
Complete and duplicated	125 (2.7%)	
Fragmented	127 (2.8%)	
Missing	204 (4.4%)	
Genome Annotation		
Number of protein-coding genes	27,420	
Number of functionally-annotated proteins	26,211	

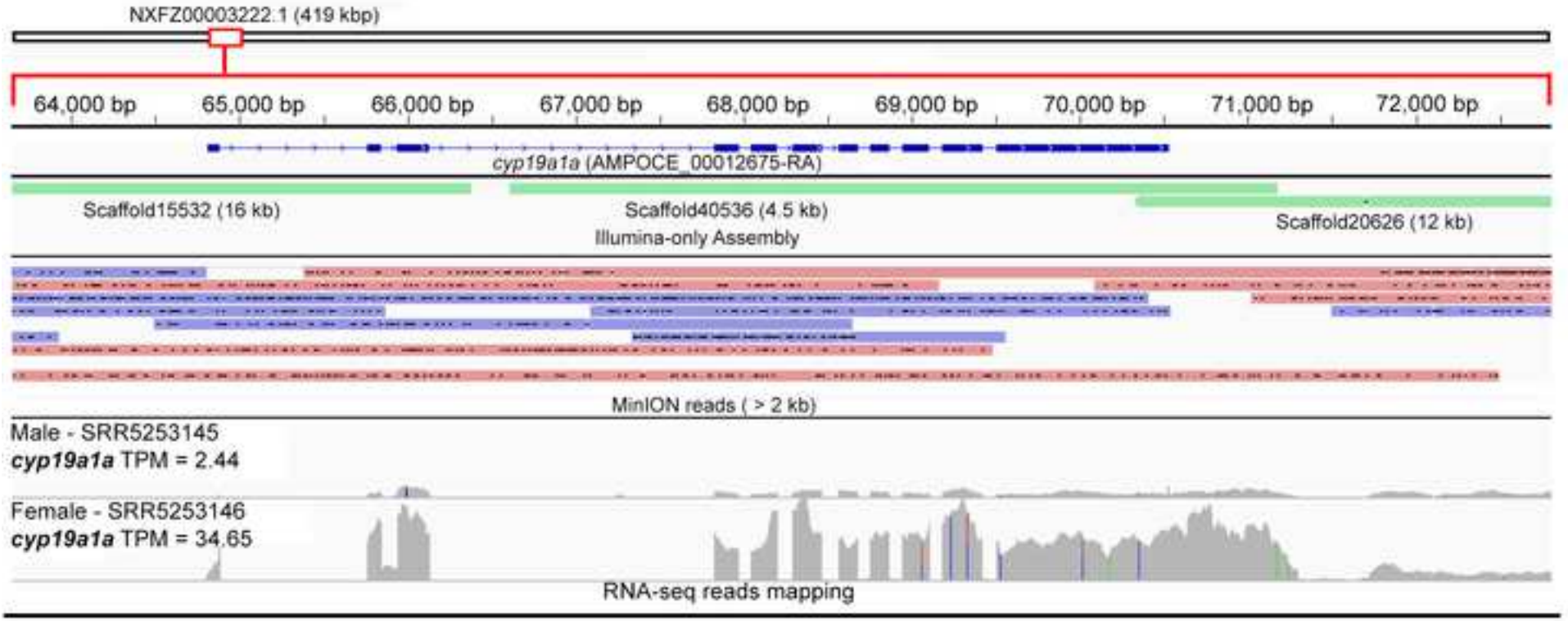
Mean protein length	514 aa
Longest protein	29,084 aa (titin protein)
Average number (length) of exon per gene	9 (355 bp)
Average number (length) of intron per gene	8 (1,532 bp)

Figure 1

Click here to download Figure Figure1.jpg

Figure 2

Click here to download Figure Figure2.tif



Supplementary Figure 1