Finding Nemo's Genes: A chromosome-scale reference assembly of the genome of the orange clownfish *Amphiprion percula*

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The Nemo Genome

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Abstract

The iconic orange clownfish, *Amphiprion percula*, is a model organism for studying the ecology and evolution of reef fishes, including patterns of population connectivity, sex change, social organization, habitat selection and adaptation to climate change. Notably, the orange clownfish is the only reef fish for which a complete larval dispersal kernel has been established and was the first fish species for which it was demonstrated that anti-predator responses of reef fishes could be impaired by ocean acidification. Despite its importance, molecular resources for this species remain scarce and until now it lacked a reference genome assembly. Here we present a *de novo* chromosome-scale assembly of the genome of the orange clownfish *Amphiprion percula*. We utilized single-molecule real-time sequencing technology from Pacific Biosciences to produce an initial polished assembly comprised of 1,414 contigs, with a contig N50 length of 1.86 Mb. Using Hi-C based chromatin contact maps, 98% of the genome assembly were placed into 24 chromosomes, resulting in a final assembly of 908.8 Mb in length with contig and scaffold N50s of 3.12 and 38.4 Mb, respectively. This makes it one of the most contiguous and complete fish genome assemblies currently available. The genome was annotated with 26,597 protein coding genes and contains 96% of the core set of conserved actinopterygian orthologs. The availability of this reference genome assembly as a community resource will further strengthen the role of the orange clownfish as a model species for research on the ecology and evolution of reef fishes.

Introduction

The orange clownfish, *Amphiprion percula*, which was immortalized in the film "Finding Nemo", is arguably the most recognized fish on Earth. It is also one of the most important species for studying the ecology and evolution of coral reef fishes. The orange clownfish is used as a model species to study patterns and processes of social organization (Buston, Bogdanowicz, Wong, & Harrison, 2007; Buston & Wong, 2014; Wong, Uppaluri, Medina, Seymour, & Buston, 2016), sex change (Buston, 2003), mutualism (Schmiege, D'Aloia, & Buston, 2017), habitat selection (Dixson et al., 2008; Elliott & Mariscal, 2001; Scott & Dixson, 2016), lifespan (Buston & García, 2007) and predator-prey interactions (Dixson, 2012; Manassa, Dixson, McCormick, & Chivers, 2013). It has been central to ground-breaking research into the scale of larval dispersal and population connectivity in marine fishes (Almany et al., 2017; Pinsky et al., 2017; Planes, Jones, & Thorrold, 2009; Salles et al., 2016) and how this influences the efficacy of marine protected areas (Berumen et al., 2012; Planes et al., 2009). It is also used to study the ecological effects of environmental disturbances in marine ecosystems (Hess, Wenger, Ainsworth, & Rummer, 2015; Wenger et al., 2014), including climate change (McLeod et al., 2013; Saenz-Agudelo, Jones, Thorrold, & Planes, 2011) and ocean acidification (Dixson, Munday, & Jones, 2010; Jarrold, Humphrey, McCormick, & Munday, 2017; Munday et al., 2009; Simpson et al., 2011). Perhaps more than any other species, the orange clownfish has become a mainstay of research into the chemical, molecular, behavioral, population, conservation and climate-change ecology of marine fishes.

The orange clownfish is one of 30 species of anemonefishes belonging to the subfamily Amphiprioninae within the family Pomacentridae (damselfishes). The two clownfishes, *A. percula* (orange clownfish or clown anemonefish) and *A. ocellaris* (false clownfish or western

clown anemonefish) form a separate clade, alongside *Premnas biaculeatus*, within the Amphiprioninae (J. Li, Chen, Kang, & Liu, 2015; Litsios, Pearman, Lanterbecq, Tolou, & Salamin, 2014; Litsios & Salamin, 2014). The two species of clownfish are easily distinguished from other anemonefishes by their bright orange body coloration and three vertical white bars. The orange clownfish and the false clownfish have similar body coloration, but largely distinct allopatric geographical distributions (Litsios & Salamin, 2014). The orange clownfish occurs in northern Australia, including the Great Barrier Reef (GBR), and in Papua New Guinea, Solomon Islands and Vanuatu, while the false clownfish occurs in the Indo-Malaysian region, from the Ryukyu Islands of Japan, throughout south-east Asia and south to north-western Australia (but not the GBR).

Like all anemonefishes, the orange clownfish has a mutualistic relationship with sea-anemones. Wild adults and juveniles live exclusively in association with a sea anemone, where they gain shelter from predators and benefit from food captured by the anemone (Fautin, 1991; Fautin & Allen, 1997; Mebs, 2009). In return, the sea-anemone benefits by gaining protection from predators (Fautin & Allen, 1997; Holbrook & Schmitt, 2005), from supplemental nutrition from the clownfish's waste (Holbrook & Schmitt, 2005) and from increased gas exchange as a result of increased water flow provided by clownfish movement and activity (Herbert, Bröhl, Springer, & Kunzmann, 2017; Szczebak, Henry, Al-Horani, & Chadwick, 2013). The orange clownfish associates with two species of anemone, *Stichodactyla gigantea* and *Heteractis magnifica* (Fautin & Allen, 1997). Clownfish social groups typically consist of an adult breeding pair and a variable number of smaller, size-ranked juveniles that queue for breeding rights (Buston, 2003). The breeding female is larger than the male. If the female disappears, the male changes sex to female and the largest non-

102 breeder matures into a breeding male. The breeding pair lays clutches of demersal eggs in
103 close proximity to their host anemone. Eggs hatch after 7-8 days and the larvae disperse into
104 the open ocean for a period of 11-12 days, at which time they return to the reef and settle to
105 an anemone.
106
107 The close association of clownfish and other anemonefishes with sea anemones makes them
108 excellent species for studying aspects of marine mutualisms and habitat selection. The easily
109 identified and delineated habitat they occupy, along with the ease with which the fish can be
110 observed in nature, makes them ideal candidates for behavioral and population ecology. The
111 unique capacity to collect juveniles immediately after they have settled to the reef from their
112 pelagic larval phase also makes them ideally suited to testing long-standing questions about
113 larval dispersal and population connectivity in reef fish populations. Using molecular
114 techniques to assign parentage between newly settled juveniles and adult anemonefishes,
115 recent studies have been able to describe for the first time the spatial scales of dispersal in reef
116 fish and its temporal consistency (Almany et al., 2017). The ability to map the connectivity of
117 clownfish populations in space and time has also opened the door to addressing challenging
118 questions about selection, fitness and adaptation in natural populations of marine fishes
119 (Pinsky et al., 2017; Salles et al., 2016). Finally, the orange clownfish is one of the relatively
120 few coral reef fishes that can easily be reared in captivity (Wittenrich, Turingan, & Creswell,
121 2007). Consequently, it has unrivalled potential for experimental manipulation to test
122 ecological and evolutionary questions in marine ecology (Dixson et al., 2014; Manassa et al.,
123 2013), including the impacts of climate change and ocean acidification (Nilsson et al., 2012).
124 Increasingly, genome-wide methods are being used to test ecological and evolutionary

125 questions and this is particularly true for coral reef species in the wake of anthropomorphic
126 climate change and its effects on these sensitive ecosystems (Stillman & Armstrong, 2015)
127
128 To date, genome assemblies of two anemonefish, *A. frenatus* (Marcionetti, Rossier, Bertrand,
129 Litsios, & Salamin, 2018) and *A. ocellaris* (Marcionetti et al., 2018), have been published.
130 Both of these were based on short-read Illumina technology with genome scaffolding
131 provided by shallow coverage of PacBio (Marcionetti et al., 2018) or Oxford Nanopore (Tan
132 et al., 2018) long reads. While the use of long reads to scaffold Illumina-based assemblies
133 improves contiguity, both genome assemblies are highly fragmented with respective contig
134 and scaffold N50s of 14.9 and 244.5 kb for *A. frenatus* and 323.6 and 401.7 kb for *A.*
135 *ocellaris*. Here we present a chromosome-scale genome assembly of the orange clownfish,
136 which was assembled using a primary PacBio long read strategy, followed by scaffolding
137 with Hi-C-based chromatin contact maps. The resulting final assembly is highly contiguous
138 with contig and scaffold N50 values of 3.12 and 38.4 Mb, respectively. This assembly will be
139 a valuable resource for the research community and will further establish the orange
140 clownfish as a model organism for genetic and genomic studies into ecological, evolutionary
141 and environmental aspects of reef fishes. To facilitate the use of this resource, we have
142 developed an integrated database, the Nemo Genome DB (http://nemogenome.org/), which
143 allows for the interrogation and mining of genomic and transcriptomic data described here.
144
145
146
147
148 **Materials and Methods**

149 Specimen collection and DNA extraction

150 Adult orange clownfish breeding pairs were collected on the northern GBR in Australia. Fish
151 were bred at the Experimental Aquarium Facility of James Cook University (JCU) and one
152 individual offspring was sacrificed at the age of 8 months. The whole brain was excised, snap
153 frozen and kept at -80°C until processing. High molecular weight DNA was extracted from
154 whole brain tissue using the Qiagen Genomic-tip 100/G extraction kit. The tissue was first
155 homogenized in lysis buffer G2 supplemented with 200 µg/mL RNase A using sterile beads
156 for 30 sec. After homogenization, proteinase K was added and the homogenate was incubated
157 at 50°C overnight. DNA extraction was then performed according to the manufacturer's
158 protocol with a final elution volume of 200 µl. DNA fragment size and quality was assessed
159 using pulsed-field gel electrophoresis. This study was completed under JCU animal ethics
160 permits A1961 and A2255.

161

162 PacBio library preparation and sequencing

163 For Pacific Biosciences (PacBio) long read sequencing, the extracted orange clownfish DNA
164 was first sheared using a g-TUBE (Covaris, MA, USA) (target size of 20 kb) and then
165 converted into SMRTbell template libraries according to the manufacturer's protocol (Pacific
166 Biosciences, CA, USA). Size selection was performed using BluePippin (Sage Science, MA,
167 USA) to generate two libraries with a minimum size of 10 and 15 kb, respectively.
168 Sequencing was performed using P6-C4 chemistry on the PacBio RS II instrument at the King
169 Abdullah University of Science and Technology (KAUST) Bioscience Core Laboratory
170 (BCL) with 360 mins movies. A total of 113 SMRT cells were sequenced.

171

172 Genome assembly

173 The genome sequence was assembled from the unprocessed PacBio reads (Table S1) using
174 the hierarchical diploid aware PacBio assembler FALCON v0.4.0 (Chin et al., 2016). To
175 obtain the optimal assembly, different parameters were tested (Table S2) to generate 12
176 candidate assemblies. The contiguity of these assemblies was assessed with QUAST v3.2
177 (Gurevich, Saveliev, Vyahhi, & Tesler, 2013), while assembly completeness was determined
178 with BUSCO v2.0 (Simão, Waterhouse, Ioannidis, Kriventseva, & Zdobnov, 2015).
179 Assembly "A7" exhibits the highest contiguity and single copy orthologous gene
180 completeness and was selected for further improvement. The FALCON_Unzip algorithm was
181 then applied to the initial A7 assembly obtain a haplotype-resolved, phased assembly, termed
182 "A7-phased". Contigs less than 20 kb in length were removed from the assembly. This phased
183 assembly was polished with Quiver to achieve final consensus sequence accuracies
184 comparable to Sanger sequencing (Chin et al., 2013) using default settings, which produced
185 the "A7-phased-polished" assembly.
186
187 Genome assembly scaffolding with chromatin contact maps
188 The flash-frozen brain tissue was sent to Phase Genomics (Seattle, WA, USA) for the
189 construction chromatin contact maps. Tissue fixation, chromatin isolation, library preparation
190 and 80-bp paired end sequencing were performed by Phase Genomics. The sequencing reads
191 were aligned to the A7-phased-polished version of the assembly with BWA (H. Li & Durbin,
192 2010) and uniquely mapping read pairs were retained. Contigs from the A7-phased-polished
193 assembly were clustered, ordered and then oriented using Proximo (Bickhart et al., 2017;
194 Burton et al., 2013), with settings as previously described (Peichel, Sullivan, Liachko, &
195 White, 2017). Briefly, contigs were clustered into chromosomal groups using a hierarchical
196 clustering algorithm based on the number of read pairs linking scaffolds, with the final

197 number of groups specified as the number of the haploid chromosomes. The haploid
198 chromosome number was set as 24, which is consistent with the observed haploid
199 chromosome number of the Amphiprioninae, as published for *A. ocellaris* (Arai, Inoue, & Ida,
200 1976), *A. frenatus*, (Molina & Galetti, 2004; Takai & Kosuga, 2007), *A. clarkii* (Arai &
201 Inoue, 1976; Takai & Kosuga, 2007), *A. perideraion* (Supiwong et al., 2015) and *A. polymnus*
202 (Tanomtong et al., 2012). After clustering into chromosomal groups, the scaffolds were
203 ordered based on Hi-C link densities and then oriented with respect to the adjacent scaffolds
204 using a weighted directed acyclic graph of all possible orientations based on the exact
205 locations of the Hi-C links between scaffolds. Gaps between contigs were represented with
206 100 Ns and the proximity-guided assembly was named "A7-PGA". Gaps in the scaffolded
207 assembly were subsequently closed using PBJelly from PBSuite v15.8.24 (English et al.,
208 2012) with the entire PacBio read dataset and Blasr (Chaisson & Tesler, 2012) (parameters: --
209 minMatch 8 --minPctIdentity 70 --bestn 1 --nCandidates 20 --maxScore -500 --nproc 32 --
210 noSplitSubreads), to give rise to the final version of the assembly, "Nemo v1".
211
212 Genome assembly validation
213 Genomic DNA was extracted from a second individual and Illumina sequencing libraries
214 were prepared using the NEBNext Ultra II DNA library prep kit for Illumina following the
215 manufacturer's protocol. Three cycles of PCR were used to enrich the library. The sequencing
216 libraries were sequenced on two lanes of a HiSeq 2500 at the KAUST BCL. A total of
217 1,199,533,204 paired reads were generated, covering approximately 181 Gb. The 151-bp
218 paired end reads were processed with Trimmomatic v0.33 to remove adapter sequences and
219 low-quality stretches of nucleotides (parameters: 2:30:10 LEADING:20 TRAILING:20
220 SLIDINGWINDOW:4:20 MINLEN:75) (Bolger, Lohse, & Usadel, 2014).

221

222 The genome assembly size was validated by comparison to a k-mer based estimate of genome
223 size. The first half of the paired-end reads of one sequencing lane (~ 25 Gb of data) was used
224 for the k-mer estimate of genome size. Firstly, KmerGenie (Chikhi & Medvedev, 2014) was
225 used to determine the optimal k-value for a k-mer based estimation. Following that, Jellyfish
226 v2.2.6 (Marçais & Kingsford, 2011) was used with k=71 to obtain the frequency distribution
227 of all k-mers with this length. The resulting distribution was analyzed with Genomescope
228 (Vurture et al., 2017) to estimate genome size, repeat content and the level of heterozygosity.
229 To further validate the assembly, we determined the proportion of trimmed Illumina short
230 reads that mapped to the Nemo v1 assembly with BWA v0.7.10 (H. Li & Durbin, 2010) and
231 SAMtools v1.1 (H. Li et al., 2009). Additionally, the completeness of the genome assembly
232 annotation as determined by the conservation of a core set of genes was measured using
233 BUSCO with default parameters.

234

235 Repeat annotation

236 A species-specific *de novo* repeat library was assembled by combining the results of three
237 distinct repeat annotation methods. Firstly, RepeatModeler v1.08 (Smit & Hubley, 2008) was
238 used to build an initial repeat library. Secondly, we used LtrHarvest (Ellinghaus, Kurtz, &
239 Willhoeft, 2008) and LTRdigest (Steinbiss, Willhoeft, Gremme, & Kurtz, 2009), both
240 accessed *via* genometools 1.5.6 (Gremme, Steinbiss, & Kurtz, 2013), with the following
241 parameters: -seed 76 -xdrop 7 -mat 2 -mis -2 -ins -3 -del -3 -mintsd 4 -maxtsd 20 -minlenltr
242 100 -maxlenltr 6000 -maxdistltr 25000 -mindistltr 1500 -similar 90. The resulting hits were
243 filtered with LTRdigest, accepting only sequences featuring a hit to one of the hidden markov
244 models in the GyDB 2.0 database. Thirdly, TransposonPSI v08222010 (Haas, 2018) was used

245 to detect sequences with similarities to known families of transposon open reading frames. To
246 remove duplicated sequences in the combined result from all three methods a clustering with
247 USEARCH (Edgar, 2010) was performed requiring at least 90% sequence identity, and only
248 cluster representatives were retained. The resulting representative sequences were classified
249 by RepeatClassifier (part of RepeatModeler), Censor v4.2.29 (Jurka, Klonowski, Dagman, &
250 Pelton, 1996) and Dfam v2.0 (Wheeler et al., 2012), and were then blasted against the
251 Uniprot/Swissprot database (release 2017_12) to obtain a unified classification. Furthermore,
252 these three classification methods and the blast result was used to filter out spurious matches
253 to protein-coding sequence. Specifically, putative repeat sequences were only retained when
254 at least one classification method recognized the sequence as a repeat and the best match in
255 Swissprot/Uniprot was not a protein-coding gene (default blastx settings). Furthermore,
256 sequences were retained if two of the three identification methods classified the sequence as
257 repeat, but the best blast hit was not a transposable element. This *de novo* library was
258 combined with the thoroughly curated zebrafish repeat library provided by Repbase v22.05
259 (Bao, Kojima, & Kohany, 2015) and this combined library was employed for repeat masking
260 in the Nemo v1 assembly using RepeatMasker (Smit, Hubley, & Green, 2010).
261
262 <u>RNA extraction, library construction, sequencing and read processing</u>
263 Tissues for RNA extraction were dissected from one eight-month old orange clownfish
264 individual. RNA was extracted from skin, eye, muscle, gill, liver, kidney, gallbladder,
265 stomach and fin tissues using the Qiagen AllPrep kit following manufacturer's instructions.
266 Sequencing libraries were prepared using the TruSeq Stranded mRNA Library Preparation kit
267 and 150 bp paired-end sequencing was performed on one lane of an Illumina HiSeq 4000
268 machine in the KAUST BCL. The RNA-seq reads were trimmed with Trimmomatic v0.33

269 (Bolger et al., 2014) (parameters: 2:30:10 LEADING:3 TRAILING:3
270 SLIDINGWINDOW:4:15 MINLEN:40) and contamination was removed with Kraken (Wood
271 & Salzberg, 2014) by retaining only unclassified reads.
272
273 Genome assembly annotation
274 After mapping the RNA-seq data with STAR v2.5.2b (Dobin et al., 2013) to the final
275 assembly, an *ab-initio* annotation with BRAKER1 v1.9 (Hoff, Lange, Lomsadze,
276 Borodovsky, & Stanke, 2016) was performed. This initial annotation identified 49,881 genes.
277 This annotation was then integrated with external evidence using the MAKER2 v2.31.8 (Holt
278 & Yandell, 2011) gene annotation pipeline. First, the transcriptome of the orange clownfish
279 was provided to MAKER2 as EST evidence in two forms, a *de novo* assembly of the
280 preprocessed RNA-seq reads obtained with Trinity v2.4.0 (Grabherr et al., 2011), and a
281 genome-guided assembly performed with the Hisat2 v2.1.0/Stringtie v1.3.3b workflow
282 (Pertea, Kim, Pertea, Leek, & Salzberg, 2016). Second, we combined the proteomes of
283 zebrafish (GCF_000002035.6_GRCz11), Nile tilapia (GCF_001858045.1_ASM185804v2)
284 and bicolor damselfish (*Stegastes partitus*) (GCA_000690725.1), together with the
285 Uniprot/Swissprot database (release 2017_12: 554,515 sequences) and the successfully
286 detected BUSCO genes to generate a reference protein set for homology based gene
287 prediction. In the initial MAKER2 run, the annotation edit distances (AED) were calculated
288 for the BRAKER1-obtained annotation, and only gene annotations with an AED of less than
289 0.1 and a corresponding protein length of greater than 50 amino acids were retained for
290 subsequent training of the gene prediction program SNAP v2013.11.29 (Korf, 2004).
291 Similarly, the AUGUSTUS v3.2.3 (Stanke et al., 2006) gene prediction program was trained
292 on 1,850 gene annotations that possessed: an AED score of less than 0.01; an initial start

293 codon, a terminal stop codon and no in-frame stop codons; more than one exon; and no
294 introns greater than 10 kb. The hidden markov gene model of GeneMark v4.32 (Ter-
295 Hovhannisyan, Lomsadze, Chernoff, & Borodovsky, 2008) was trained by BRAKER1. The
296 final annotation was then obtained in the second run of MAKER2 with the trained models for
297 SNAP, GeneMark, and AUGUSTUS. InterProScan 5 was then used to obtain the Pfam
298 protein domain annotations for all genes. The standard gene builds were then generated. The
299 output was filtered to include all annotated genes with evidence (AED less than 1) or with a
300 Pfam protein domain, as recommended (Campbell, Holt, Moore, & Yandell, 2014).
301
302 Functional annotation
303 The protein sequences produced from the genome assembly annotation were aligned to the
304 UniProtKB/Swiss-Prot database (release 2017_12) with blastp v2.2.29 (parameters: -outfmt 5
305 -evalue 1e-3 -word_size 3 -show_gis -num_alignments 20 -max_hsps 20) and protein
306 signatures were annotated with InterProScan 5. The results were then integrated with
307 Blast2GO v4.1.9 (Gotz et al., 2008).
308
309 Genome assembly comparisons
310 For genome assembly comparisons, we compared the Nemo v1 genome assembly to the 26
311 previously reported fish chromosome-scale genome assemblies (Table S3). Comparisons were
312 made for genome assembly contiguity and completeness. Contig N50 values are reported for
313 the scaffold-scale versions of each assembly and are taken from the indicated publication
314 (Table S3), database description (Table S3) or were generated with the Perl
315 assemblathon_stats_2.pl script (Bradnam et al., 2013). Genome assembly completeness was
316 assessed by determining the proportion of the genome size that is contained within the

317 chromosome content of each assembly. It should be noted that this comparison is relative to
318 the estimated genome size and not the published assembly size. The estimated genome size
319 was taken as either the published estimated genome size in the relevant paper (Table S3), or
320 from the Animal Genome Size Database (Gregory, 2018). Where possible, k-mer derived or
321 flow cytometry-based estimates of genome size were used. Before calculation, we remove
322 stretches of Ns from the genome assemblies as these are used to arbitrarily space scaffolds
323 and do not contain actual genome information. However, this step was not possible for the
324 Asian arowana, southern platyfish, yellowtail or croaker genomes as the chromosome-scale
325 assemblies have not been made publicly available. Genome assembly completeness was
326 determined with BUSCO (Simão et al., 2015) using the Actinopterygii set of 4,584 genes and
327 the AUGUSTUS zebrafish gene model provided with the software.
328
329 Gene homology
330 To investigate the gene space of the orange clownfish genome assembly, we used
331 OrthoFinder v1.1.4 (Emms & Kelly, 2015) to identify orthologous gene relationships between
332 the orange clownfish and four related fish species. The following four fish species were
333 utilized in addition to the orange clownfish: Asian seabass
334 GCF_001640805.1_ASM164080v1 (45,223 sequences), Nile tilapia
335 GCF_001858045.1_ASM185804v2 (58,087 sequences), southern platyfish
336 GCF_000241075.1_*Xiphophorus_maculatus*-4.4.2 (23,478 sequences), and zebrafish
337 GCF_000002035.6_GRCz11 (52,829 sequences). The longest isoform of each gene was
338 utilized in the analysis, which corresponded to 25,050, 28,497, 23,043, and 32,420 sequences,
339 respectively. 26,597 sequences were used for the orange clownfish. These protein sequences
340 were reciprocally blasted against each other and clusters of orthologous genes were then

341 defined using OrthoFinder with default parameters. As part of OrthoFinder, the concatenated
342 sequences of single-copy orthologs present in all species were then used to construct a
343 phylogenetic tree, which was rooted using STRIDE (Emms & Kelly, 2017).
344
345 Database system architecture and software
346 The Nemo Genome DB database (http://nemogenome.org) was implemented on a UNIX
347 server with CentOS version 7, Apache web server and MySQL Database server. JBrowse
348 (Buels et al., 2016) was employed to visualize the genome assembly and genomic features
349 graphically and interactively. JavaScript was adopted to implement client-side rich
350 applications. The JavaScript library, jQuery (http://jquery.com) was employed. Other
351 conventional utilities for UNIX computing were appropriately installed on the server if
352 necessary. All of the Nemo Genome DB resources are stored on the server and are available
353 through HTTP access.
354
355
356 **Results and Discussion**
357 Sequencing and assembly of the orange clownfish genome
358 Genomic DNA of an individual orange clownfish (Fig. 1A) was sequenced with the PacBio
359 RS II platform to generate 1,995,360 long reads, yielding 113.8 Gb, which corresponds to a
360 121-fold coverage of the genome (Table S1). After filtering with the read pre-assembly step
361 of the Falcon assembler, 5,764,748 reads, covering 54.3 Gb and representing a 58-fold
362 coverage of the genome, were available for assembly.
363

364 To optimize the assembly parameters, we performed 12 trial assemblies using a range of
365 parameters for different stages of the Falcon assembler (Table S2). The assembly quality was
366 assessed by considering assembly contiguity (contig N50 and L50), total assembly size, and
367 also gene completeness (BUSCO) (Table 1). Assembly A7 exhibited the highest contig N50
368 (1.80 Mb), lowest contig L50 (138 contigs), lowest number of missing BUSCO genes (132)
369 and is only slightly surpassed in the longest contig metric (15.8 Mb) by the highly similar
370 assemblies A8 and A9 (16.5 Mb) (Table 1).
371
372 Genome assemblies represent a mixture of the two possible haplotypes of a diploid individual
373 at each locus. This collapsing of haplotypes may result in a loss of important sequence
374 information. However, diploid-aware assembly algorithms such as the Falcon_Unzip
375 assembler are designed to detect single nucleotide polymorphisms (SNPs) as well as structural
376 variations and to use this information to phase ("unzip") heterozygous regions into distinct
377 haplotypes (Chin et al., 2016). This procedure results in a primary assembly and a set of
378 associated haplotype contigs (haplotigs) capturing the divergent sequences. Having
379 established the parameter set that gave the best assembly metrics with Falcon, we used
380 Falcon_Unzip to produce a phased assembly ("A7-phased") of the orange clownfish (Table
381 2). The phased assembly was 905.0 Mb in length with a contig N50 of 1.85 Mb. As has been
382 seen in previous genome assembly projects (Chin et al., 2016), Falcon_Unzip produced a
383 smaller assembly with fewer contigs than the assembly produced by Falcon (Table 2). The
384 phased primary assembly was then polished with Quiver, which yielded an assembly ("A7-
385 phased-polished") with 1,414 contigs spanning 903.6 Mb with an N50 of 1.86 Mb (Table 2).
386 This polishing step closed 91 gaps in the assembly and improved the N50 by approximately
387 14.3 kb. After polishing of the "unzipped" A7-phased-polished assembly, 9,971 secondary

388 contigs were resolved, covering 340.1 Mb of the genome assembly. The contig N50 of these
389 secondary contigs was 38.2 kb, with over 99% of them being longer than 10 kb in size.
390 Relative to the 903.6 Mb A7-phased-polished primary contig assembly, the secondary contigs
391 covered 38% of the assembly size. To the best of our knowledge, this is the first published
392 fish genome assembly that has been resolved to the haplotype level with Falcon_Unzip.
393
394 Scaffolding of the orange clownfish genome assembly into chromosomes
395 To build a chromosome-scale reference genome assembly of the orange clownfish, chromatin
396 contact maps were generated by Phase Genomics (Fig. S1). Scaffolding was performed by the
397 Proximo algorithm (Bickhart et al., 2017; Burton et al., 2013) on the A7-phased-polished
398 assembly using 231 million Hi-C-based paired-end reads to produce the proximity guided
399 assembly "A7-PGA" (Table 2). The contig clustering allowed the placement of 1,073 contigs
400 into 24 scaffolds (chromosomes) with lengths ranging from 23.4 to 45.8 Mb (Tables 2 and 3).
401 While only 76% of the contigs were assembled into chromosome clusters, this corresponds to
402 98% (885.4 Mb) of total assembly length and represents 95% of the estimated genome size of
403 938.9 Mb (Tables 2 and 3). This step substantially improved the overall assembly contiguity,
404 raising the N50 20-fold from 1.86 to 38.1 Mb.
405
406 A quality score for the order and orientation of contigs within the A7-PGA assembly was
407 determined. This metric is based on the differential log-likelihood of the contig orientation
408 having produced the observed log-likelihood, relative to its neighbors (Burton et al., 2013).
409 The orientation of a contig was deemed to be of high quality if its placement and orientation,
410 relative to neighbors, was 100 times more likely than alternatives (Burton et al., 2013). In A7-
411 PGA, the placements of 524 (37%) of the scaffolds were deemed to be of high quality,

412 accounting for 775.5 Mb (87%) of the scaffolded chromosomes, indicating the robustness of
413 the assembly.
414
415 A final polishing step was performed with PBJelly to generate the final Nemo v1 assembly.
416 This polishing step closed 369 gaps, thereby improving the contig N50 by 68% and increasing
417 the total assembly length by 5.21 Mb (Tables 2 and 3). The length of each chromosome was
418 increased, with a range of 23.7 to 46.1 Mb (Fig. 1B). Gaps were closed in each chromosome
419 except for chromosome 14, leaving an average of only 28 gaps per chromosome (Table 3).
420 The final assembly is 908.9 Mb in size and has contig and scaffold N50s of 3.12 and 38.4 Mb,
421 respectively. The assembly is highly contiguous as can be observed by the fact that 50% of
422 the genome length is contained within the largest 84 contigs. 890.2 Mb (98%) of the genome
423 assembly size was scaffolded into 24 chromosomes, with only 18.8 Mb of the assembly
424 failing to be grouped. The 18.8 Mb of unscaffolded assembly is comprised of 341 contigs
425 with a contig N50 of only 57.8 kb.
426
427 Validation of the orange clownfish genome assembly size
428 The final assembly size of 908.9 Mb is consistent with the results of a Feulgen image analysis
429 densitometry-based study, which determined a C-value of 0.96 pg and thus a genome size of
430 938.9 Mb for the orange clownfish (Hardie & Hebert, 2004). Furthermore, our assembly size
431 is in keeping with estimates of genome size for other fish of the *Amphiprion* genus, which
432 range from 792 to 1193 Mb (Gregory, 2018). We additionally validated the observed
433 assembly size by using a k-mer based approach. Specifically, the k-mer coverage and
434 frequency distribution were plotted and fitted with a four-component statistical model with
435 GenomeScope (Fig. S2A). This allowed us to generate an estimate of genome size as well as

436 the repeat content and level of heterozygosity. However, varying the k-value from the
437 recommended value of 21 up to 27 yielded a corresponding increase of the estimated genome
438 size. We therefore used KmerGenie to determine the optimal k-mer length of 71 to capture the
439 available sequence information. The utilization of small k-values might partially explain the
440 reported tendency of GenomeScope to underestimate the genome size (Vurture et al., 2017).
441 The final estimate of the haploid genome length by k-mer analysis was 906.6 Mb, with 732.8
442 Mb (80%) of unique sequence and a repeat content of 173.8 Mb (19%). Furthermore, the
443 estimated heterozygosity level of 0.12% is low considering that an F1 offspring of wild
444 caught fish was sequenced (Fig. S2B). While the short-read k-mer based genome size
445 estimate of 906.6 Mb matches the final assembly size of 908.9 Mb very well, the C-value
446 derived genome size estimate is slightly larger (938.9 Mb). As an additional validation of the
447 accuracy of the genome assembly, we mapped the trimmed Illumina short reads to the Nemo
448 v1 assembly and observed that 95% of the reads mapped to the assembly and that 84% of the
449 reads were properly paired.
450
451 Based on the C-value derived genome size estimate, there is approximately 29.9 Mb (3.3%)
452 of sequence length absent from our genome assembly. It seems likely that our assembly is
453 nearly complete for the euchromatic regions of the genome given our assessment of genome
454 size and gene content completeness. However, genomic regions such as the proximal and
455 distal boundaries of euchromatic regions contain heterochromatic and telomeric repeats,
456 respectively, are refractory to currently available sequencing techniques and are typically
457 absent from genome assemblies (Bickhart et al., 2017; Hoskins et al., 2007).
458
459 Chromosome-scale fish genome assembly comparisons

460 To date, chromosome-scale genome assemblies have been released for 26 other fish species
461 (Table S3). Here, we present the first chromosome-scale assembly of a tropical coral reef fish,
462 the orange clownfish. As a measure of genome assembly quality, we assessed the contiguity
463 and completeness of these 27 chromosome-scale genome assemblies. We investigated
464 genome contiguity with the contig N50 metric and characterized genome completeness for
465 each genome assembly by calculating the proportion of the estimated genome size that was
466 assigned to chromosomes. As shown in Fig. 1C, the orange clownfish genome assembly is
467 highly contiguous, with a scaffold-scale contig N50 of 1.86 Mb, which is only surpassed by
468 the contig N50 of the Nile tilapia genome assembly. Interestingly, even though different
469 assembler algorithms were utilized, the three genome assemblies based primarily on long read
470 PacBio technology were the most contiguous, with only Nile tilapia (3.09 Mb, Canu), orange
471 clownfish (1.86 Mb, Falcon) and Asian seabass (1.19 Mb, HGAP) genome assemblies
472 yielding contig N50s in excess of 1 Mb.
473
474 While the use of long read sequencing technologies facilitates the production of highly
475 contiguous genome assemblies, scaffold sizes are still much shorter than the length of the
476 underlying chromosomes. The use of further scaffolding technologies such as genetic linkage
477 maps, scaffolding based on synteny with genome assemblies from related organisms, as well
478 as *in vitro* and *in vivo* Hi-C based methods has allowed for the production of assemblies with
479 chromosome-sized scaffolds. Here, the use of Hi-C based chromatin contact maps allowed for
480 the placement of 98% of the Nemo v1 assembly length (890.2 of 908.9 Mb) into
481 chromosomes, yielding a final assembly with a scaffold N50 of 38.4 Mb. This corresponds to
482 95% of the estimated genome size (938.9 Mb), which suggests that the Nemo v1 assembly is
483 one of the most complete fish genome assemblies published to date (Fig. 1C). Only the

484 zebrafish (94%) and Atlantic cod (91%) genome assemblies had a comparably high
485 proportion of their estimated genome sizes scaffolded into chromosome-length scaffolds (Fig.
486 1C). It is likely that the use of both PacBio long reads and Hi-C based chromatin contact maps
487 contributed to the very high proportion of the orange clownfish genome that we were able to
488 both sequence and assemble into chromosomes.
489
490 While assembly contiguity is important, genome completeness with respect to gene content is
491 also vital for producing a genome assembly that will be utilized by the research community.
492 We evaluated the completeness of the 27 chromosome-scale assemblies with BUSCO and the
493 Actinopterygii lineage, which encompasses 4,584 highly-conserved genes. When ranked by
494 the total of complete (single copy and duplicate) genes, the orange clownfish assembly is the
495 second most complete, with 4,456 (97.2%) of the orthologs identified (Fig. 2). The top ranked
496 assembly, Nile tilapia, contains only 9 more of the core set of orthologs such that it contains
497 4,465 of the orthologs (97.4%). While the assemblies based on PacBio long read technology
498 are again amongst the most complete, it should also be noted that most of the assemblies
499 analyzed showed a very high level of completeness.
500
501 Genome annotation
502 To annotate repetitive sequences and transposable elements, we constructed an orange
503 clownfish-specific library by combining the results of Repeatmodeler, LTRharvest and
504 TransposonPSI. Duplicate sequences were removed and false positives were identified using
505 three classification protocols (Censor, Dfam, RepeatClassifier) as well as comparisons to
506 Uniprot/Swissprot databases. After these filtering steps, we identified 21,644 repetitive
507 sequences. These sequences, in combination with the zebrafish library of RepBase, were then

508 used for genome masking with RepeatMasker. This lead to a total of 28% of the assembly
509 being identified as repetitive (Fig. 3A and Table S4). It was observed that there is a general
510 trend for increased repeat density towards the ends of chromosome arms (Fig. 3B and S2).
511 The total fraction of repetitive genomic sequence is in good agreement with other related fish
512 species (Chalopin, Naville, Plard, Galiana, & Volff, 2015). Similarly, the high fraction of
513 DNA transposons (~10%) is in line with DNA transposon content in other fish species
514 (Chalopin et al., 2015) but is substantially higher that what has been reported in mammals
515 (~3%) (Chalopin et al., 2015; Lander et al., 2001).
516
517 Following the characterization of repetitive sequences in the Nemo v1 genome assembly,
518 gene annotation was performed with the BRAKER1 pipeline, which trained the AUGUSTUS
519 gene predictor with supplied RNA-seq data, and a successive refinement with the MAKER2
520 pipeline. We provided BRAKER1 with mapped RNA-seq data from 10 different tissues. This
521 initial annotation comprised 49,881 genes with 55,273 transcripts. The gene finder models of
522 SNAP and AUGUSTUS were refined based on the initial annotation, and MAKER2 was then
523 used to improve the annotation using the new models and the available protein homology and
524 RNA-seq evidence. The resulting annotation contained 26,606 genes and 35,498 transcripts,
525 which feature a low mean AED of 0.12, indicating a very good agreement with the provided
526 evidence. After retaining only genes with evidence support (AED of less than 1) or an
527 annotated Pfam protein domain, the filtered annotation was comprised of 26,597 genes,
528 corresponding to 35,478 transcripts (Table 4). This result is broadly consistent with the
529 average number of genes (23,475) found in the 22 diploid fish species considered in this study
530 (Table S3). Compared to the initial annotation, genes in the final annotation are 61% longer
531 (13,049 bp) and encode mRNAs that are 80% longer (17,727 bp). The proportion of the

532 genome that is covered by coding sequences also increased to 8.1% in the final annotation.
533 Together with the observed reduction in the gene number by 47%, this indicates a substantial
534 reduction of likely false positive gene annotations of short length and/or few exons. The gene
535 density across the 24 chromosomes of our assembly varied from 23.6 genes/Mb (chromosome
536 21) to 36.5 genes/Mb (chromosome 14), with a genome-wide average of one gene every 29.7
537 Mb (Table 3). The spatial distribution of genes across all 24 chromosomes is relatively even
538 (Fig. 1B), with regions of very low gene density presumably corresponding to centromeric
539 regions. We observed that the longest annotated gene was APERC1_00006329 (26.5 kb),
540 which encodes the extracellular matrix protein FRAS1, while the gene coding for the longest
541 protein sequence was APERC1_00011517, which codes for the 18,851 amino acid protein,
542 Titin. Functional annotation was carried out using Blast2GO and yielded annotations for
543 22,507 genes (85%) after aligning the protein sequences to the UniProt/Swissprot database
544 and annotating protein domains with InterProScan.
545
546 Identification of orange clownfish-specific genes
547 To investigate the gene space of the orange clownfish relative to other fishes, we used
548 OrthoFinder v1.1.4 (Emms & Kelly, 2015) to identify orthologous relationships between the
549 protein sequences of the orange clownfish and four other fish species (Asian seabass, Nile
550 tilapia, southern platyfish and zebrafish) from across the teleost phylogenetic tree (Betancur-
551 R. et al., 2013). The vast majority of sequences (89%) could be assigned to one of 19,838
552 orthogroups, with the remainder identified as “singlets” with no clear orthologs. We observed
553 a high degree of overlap of protein sequence sets between all five species, with 75% of all
554 orthogroups (14,783) shared amongst all species (Fig. 4A). The proteins within these
555 orthogroups presumably correspond to the core set of teleost genes. Of the 14,783

556 orthogroups with at least one sequence from each species, a subset of 8,905 orthogroups
557 contained only a single sequence from each species. The phylogeny obtained from these
558 single-copy orthologous gene sequences (Fig. 4B) is consistent with the known phylogenetic
559 tree of teleost fishes (Betancur-R. et al., 2013). Interestingly, we identified a total of 4,429
560 sequences that are specific to the orange clownfish, 2,293 (49%) of which possess functional
561 annotations (Fig. 4A). Future investigations will focus on the characterization of these unique
562 genes and what roles they may play in orange clownfish phenotypic traits.
563
564
565 **Conclusion**
566 Here, we present a reference-quality genome assembly of the iconic orange clownfish, *A.*
567 *percula*. We sequenced the genome to a depth of 121X with PacBio long reads and performed
568 a primary assembly with these reads utilizing the Falcon_Unzip algorithm. The primary
569 assembly was polished to yield an initial assembly of 903.6 Mb with a contig N50 value of
570 1.86 Mb. These contigs were then assembled into chromosome-sized scaffolds using Hi-C
571 chromatin contact maps, followed by gap-filling with the PacBio reads, to produce the final
572 reference assembly, Nemo v1. The Nemo v1 assembly is highly contiguous, with contig and
573 scaffold N50s of 3.12 and 38.4 Mb, respectively. The use of Hi-C chromatin contact maps
574 allowed us to scaffold 890.2 Mb (98%) of the 908.2 Mb final assembly into the 24
575 chromosomes of the orange clownfish. An analysis of the core set of Actinopterygii genes
576 suggests that our assembly is nearly complete, containing 97% of the core set of highly
577 conserved genes. The Nemo v1 assembly was annotated with 26,597 genes with an average
578 AED score of 0.12, suggesting that most gene models are highly supported.
579

580 The high-quality Nemo v1 reference genome assembly described here will facilitate the use of
581 this now genome-enabled model species to investigate ecological, environmental and
582 evolutionary aspects of reef fishes. To assist the research community, we have created the
583 Nemo Genome DB database, http://nemogenome.org/ (Fig. 5), where researchers can access,
584 mine and visualize the genomic and transcriptomic resources of the orange clownfish.
585
586

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598

599

600 **Author contributions**

601 R.L. and D.J.L. designed and performed the computational analysis. R.L., T.R., C.S. and
602 D.J.L. interpreted the results. H.O., K.M. and T.G. created the database. C.T.M. and S.F.
603 produced sequencing libraries. R.L., D.J.L, T.R., P.L.M., M.L.B., M.A. and D.J.M. wrote the
604 manuscript and all authors approved the final version. T.R. supervised the project.

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609

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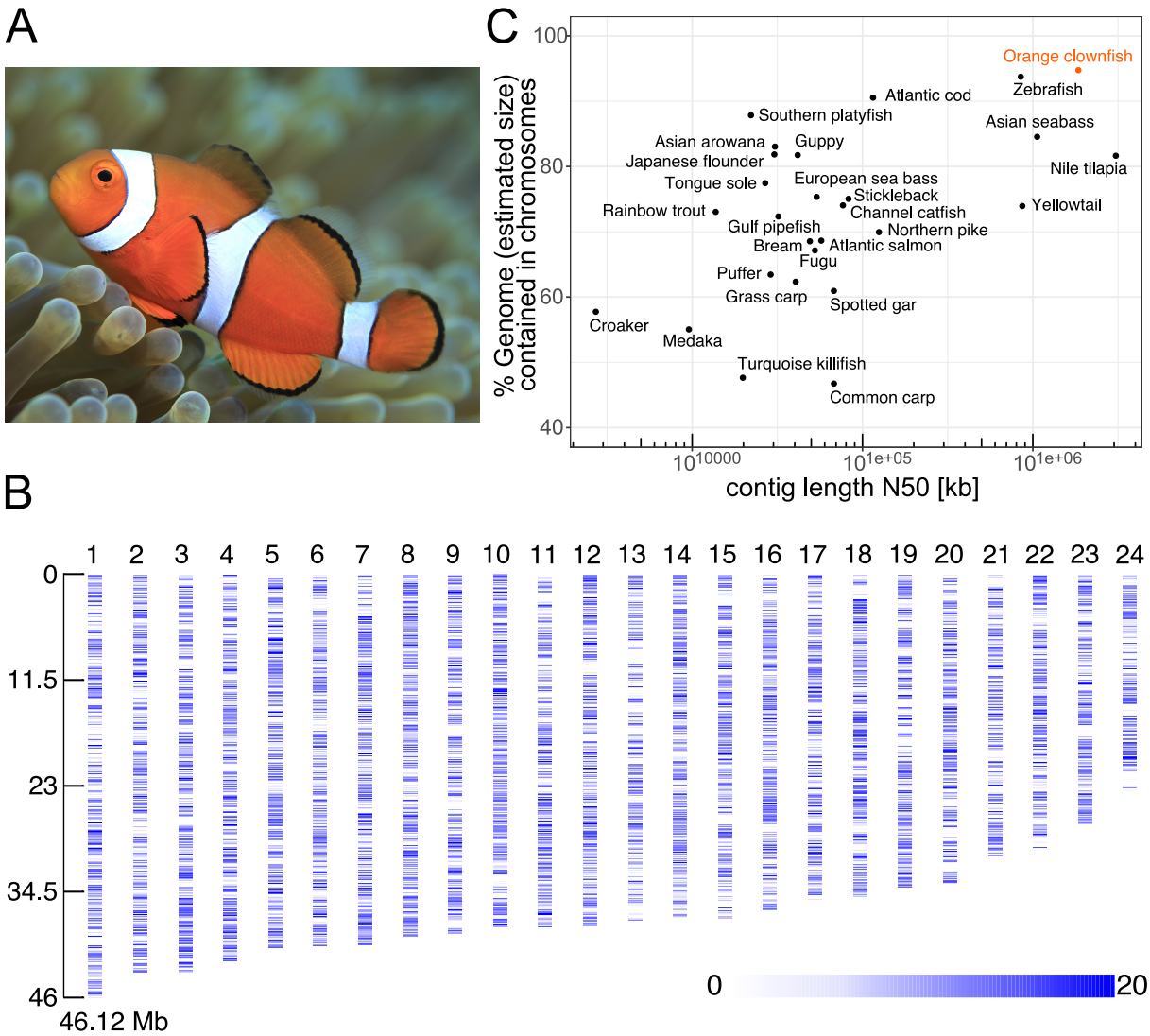
853 **Data Accessibility**

854 The assembled and annotated genome as well as the raw PacBio reads and Illumina reads are
855 available at the Nemo Genome DB (http://nemogenome.org). Furthermore, the assembled
856 genome will be available on GenBank as BioProject PRJNA436093 and BioSample accession
857 SAMN08615572. All raw sequencing data described in this study will be available via the
858 NCBI Sequencing Read Archive.

859 **Tables and Figures**

860



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862 **Fig. 1 (A)** The iconic orange clownfish (*A. percula*). **(B)** Gene density on the 24
863 chromosomes, plotted in 100 kb windows. Chromosomes are ordered by size, as indicated on
864 the left axis in Mb. **(C)** Contiguity (x-axis) and genome assembly completeness (y-axis) of the
865 orange clownfish, and the 26 previously published, chromosome-scale fish genome
866 assemblies. Details and statistics of the 27 assemblies are presented in Table S3.

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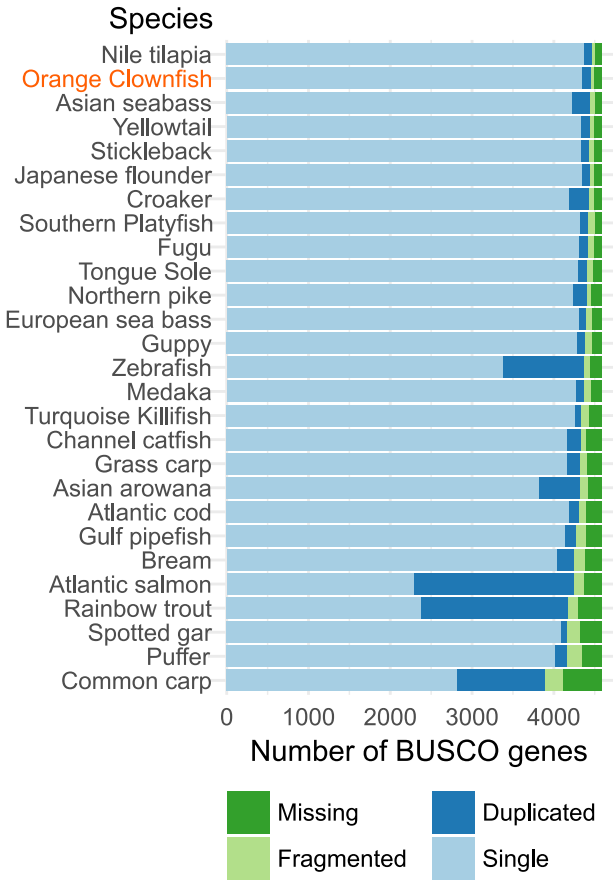

Fig. 2 Genome assembly completeness of all published chromosome-scale fish genome assemblies, as measured by the proportion of the BUSCO set of core genes detected in each assembly. Genome assemblies on the y-axis are sorted by the sum of single copy and duplicated BUSCO genes.

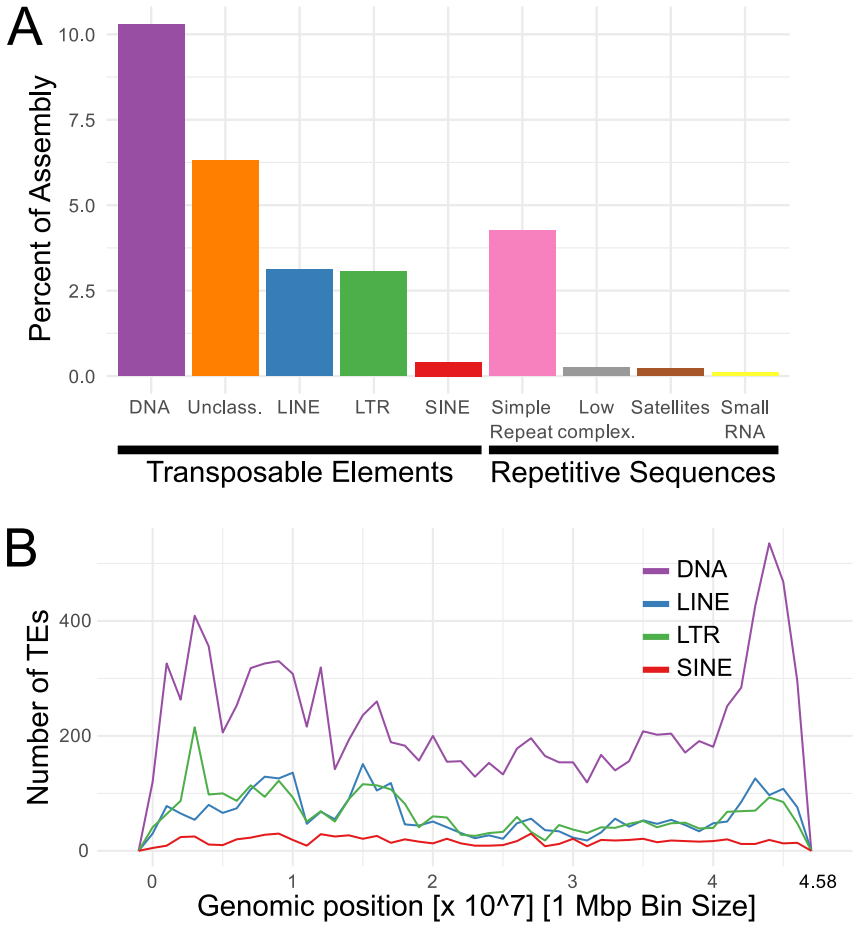

Fig. 3 Repeat content of the orange clownfish genome assembly. **(A)** Repeat content of the whole genome as classified into transposable elements and repetitive sequences. **(B)** Spatial distribution of the four main identified classes of transposable elements on chromosome 1. Transposable element spatial distribution for chromosomes 2-24 is show in Fig S2. Detailed transposable element content is shown in Table S4.

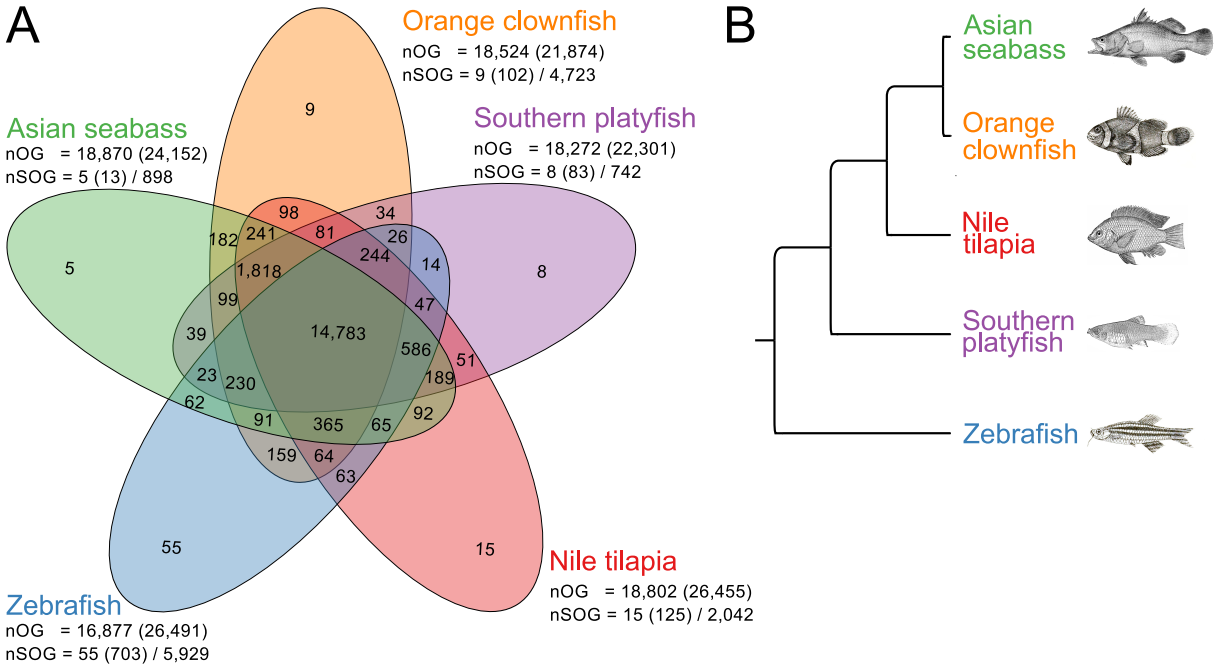

Fig. 4 (A) The overlap of orthologous gene families of the orange clownfish, southern platyfish, Nile tilapia, zebrafish and Asian seabass. The total number of orthogroups (nOG) followed by the number of genes assigned to these groups is provided below the species name. The number of species-specific orthogroups (nSOG) and the respective number of genes is also indicated, followed by the number of genes not assigned to any orthogroups. **(B)** The inferred phylogenetic tree based on the ortholog groups that contain a single gene from each species.

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B

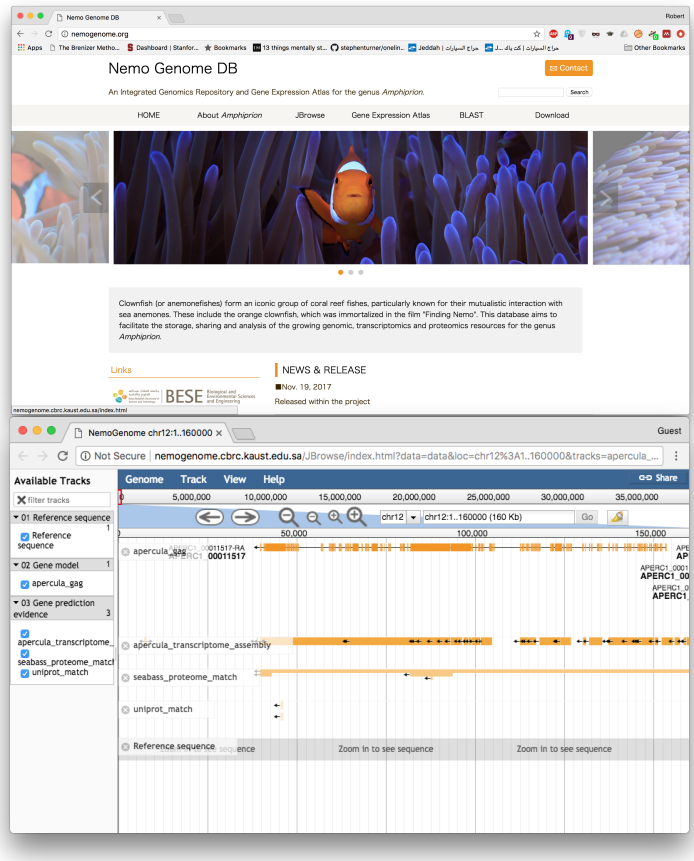

Fig. 5 (A) Front page of the Nemo Genome DB database, which is a portal to access the data described in this manuscript and is accessible at www.nemogenome.org. **(B)** Genome viewer representation of the Titin gene.

916 **Table 1** Contig statistics for the preliminary candidate-assemblies

Assembly	Length (Mb)	Number	N50 (Mb)	L50	Longest (Mb)	Missing genes (number (%))
A1	950.4	4,874	1.024	254	9.59	148 (3.23)
A2	945.4	4,374	1.040	251	6.67	156 (3.30)
A3	926.5	3,629	1.070	236	7.21	140 (3.05)
A4	921.8	2,829	1.380	184	8.16	134 (2.92)
A5	883.9	1,017	1.469	167	10.24	146 (3.18)
A6	902.2	2,204	1.401	174	12.38	134 (2.92)
A7	920.7	2,473	1.801	138	15.84	132 (2.88)
A8	924.6	2,629	1.742	143	16.51	139 (3.03)
A9	924.9	2,638	1.742	143	16.51	140 (3.05)
A10	917.1	2,368	1.648	140	10.21	146 (3.18)
A11	899.9	2,049	1.571	160	9.07	151 (3.29)
A12	908.8	2,086	1.602	142	10.21	143 (3.12)

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925 **Table 2** Assembly statistics of the orange clownfish genome assemblies
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	A7	A7-phased	A7-phased-polished	A7-PGA	Nemo v1
Technology					
Falcon	✓	-	-	-	-
Falcon_Unzip	-	✓	✓	✓	✓
PacBio	✓	✓	✓	✓	✓
Quiver	-	-	✓	✓	✓
Hi-C maps	-	-	-	✓	✓
PBJelly	-	-	-	-	✓
Contigs					
Length (Mb)	920.7	905.0	903.6	903.6	908.9
Number	2,473	1,505	1,414	1,414	1,045
N50 length (Mb)	1.80	1.85	1.86	1.86	3.12
L50 count	138	135	134	134	84
Longest (Mb)	15.84	15.83	15.85	15.9	16.6
No. Scaffolded	-	-	-	1,073	704
Scaffolds					
Length (Mb)	-	-	-	903.7	908.9
Number	-	-	-	365	365
N50 length (Mb)	-	-	-	38.1	38.4
L50 count	-	-	-	12	12
Longest (Mb)	-	-	-	45.8	46.1
Ns	-	-	-	104,900	32,395
Number of gaps	-	-	-	1,049	680
Chromosomes					
Length in chr (Mb)	-	-	-	885.4	890.2
% assembly in chr	-	-	-	98.0%	97.9%
% assembly not in chr	-	-	-	2.0%	2.1%
% of predicted genome size in chr	-	-	-	94.3%	94.8%

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928 * Predicted genome size is 938.88 Mb (Hardie & Hebert, 2004).
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930 **Table 3** Chromosome metrics before and after polishing of the final assembly

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Chromosome	A7-PGA assembly		Nemo v1 assembly			
	Contigs	Length (Mb)	Contigs	Length (Mb)	Genes	Gene density (genes/Mb)
1	57	45.8	31	46.1	1,091	23.8
2	41	43.3	31	43.4	1,132	26.1
3	55	43.2	28	43.4	1,395	32.3
4	47	42.0	29	42.2	1,259	30.0
5	32	40.5	31	40.6	1,303	32.2
6	44	40.4	24	40.6	1,337	33.1
7	37	40.2	32	40.4	1,324	32.9
8	42	39.3	26	39.4	1,276	32.5
9	47	39.0	25	39.2	1,083	27.8
10	55	38.3	38	38.6	1,339	35.0
11	40	38.3	23	38.5	1,037	27.1
12	48	38.1	23	38.4	1,067	28.0
13	30	37.6	20	37.7	1,014	27.0
14	33	37.3	33	37.4	1,362	36.5
15	45	37.3	22	37.4	1,091	29.2
16	77	36.3	50	36.6	1,018	28.0
17	35	35.2	23	35.4	987	28.0
18	40	34.9	32	35.1	1,126	32.3
19	53	34.0	35	34.2	1,062	31.2
20	46	33.4	31	33.7	1,132	33.9
21	40	30.7	21	30.8	725	23.6
22	29	29.6	20	29.8	786	26.6
23	32	27.2	23	27.4	904	33.2
24	68	23.4	53	23.7	723	30.9
In chr:	1,073	885.4	704	890.2	26,309	Ave: 29.7
Not in chr:	341	18.4	341	18.8	288	15.3
Total:	1,414	903.7	1,045	908.8	26,597	Ave: 29.3

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933 **Table 4** Gene annotation statistics

	Initial BRAKER1	Final MAKER2
Genes	49,881	26,597
mRNAs	55,273	35,478
Exons	391,637	463,688
Introns	336,364	428,210
CDSs	55,273	35,478
Overlapping genes	2,407	1,852
Contained genes	744	463
Longest gene	264,684	264,684
Longest mRNA	264,684	264,684
Mean gene length	8,097	13,049
Mean mRNA length	9,841	17,727
% of genome covered by genes	44.4	38.2
% of genome covered by CDS	7.5	8.1
Exons per mRNA	7	13
Introns per mRNA	6	12
BUSCO		
Completeness	95.94%	96.25%
Complete	4,398	4,412
Single-Copy	3,588	3,888
Duplicated	810	524
Fragmented	138	96
Missing	48	76
Total	4,584	4,584

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