Long-read sequence capture elucidates the evolution of the hemoglobin gene clusters in codfishes

Siv Nam Khang Hoff*[a], Helle T. Baalsrud*[a‡], Ave Tooming-Klunderud[a], Morten Skage[a], Todd Richmond[b], Gregor Obernosterer[c], Reza Shirzadi[d], Ole Kristian Tørresen[a], Kjetill S. Jakobsen[a] and Sissel Jentoft[a‡]

[a] Centre for Ecological and Evolutionary Synthesis, Department of Biosciences, University of Oslo, Oslo, Norway
[b] Roche NimbleGen Inc., Madison, WI, USA
[c] Roche Diagnostics, Mannheim, Germany
[d] Roche Diagnostics, Oslo, Norway

* these authors contributed equally to this work

[‡] Correspondence to: h.t.baalsrud@ibv.uio.no, sissel.jentoft@ibv.uio.no

Abstract

Combining high-throughput sequencing with targeted sequence capture has become an attractive tool to study specific genomic regions of interest. Most studies have so far focused on the exome using short-read technology. This approach does not capture intergenic regions needed to reconstruct genomic organization, including regulatory regions and gene synteny. In this study, we demonstrate the power of combining targeted sequence capture with long-read sequencing technology, leading to the successful sequencing and assembling of the two hemoglobin (Hb) gene clusters LA (~100kb) and MN (~200kb) across eight species of codfishes that are separated by up to 70 million years of evolution. The highly continuous assemblies – capturing both intergenic and coding sequences – revealed overall conserved genetic organization and synteny of the *Hb* genes within this lineage, yet with several, lineage-specific gene duplications. Moreover, for some of the species examined we identified amino acid substitutions at two sites in the *Hbb1* gene as well as length polymorphisms in its regulatory region, which has previously been linked to temperature adaptation in Atlantic cod populations. Taken together, our study highlights the efficiency of targeted long-read capture for comparative genomic studies by shedding light on the evolutionary history of the *Hb* gene family across the highly divergent group of codfishes.

Key words: Targeted sequence capture, comparative genomics, Gadiformes, PacBio sequencing, teleosts

Article summary

Hemoglobins (Hbs) are key respiratory proteins in most vertebrates. In fishes, Hbs are shown to be of great importance for ecological adaptation, as environmental factors including temperature, directly influences the solubility of O_2 in surrounding waters as well as the ability of Hb to bind O_2 at respiratory surfaces.

We here combine targeted sequence capture and long-read sequencing to reconstruct and resolve the organization of *Hb* genes and their flanking genes in a selection of codfishes, inhabiting different environmental conditions. Our results shed light on the evolutionary history of *Hb* genes across species separated up to 70 million years of evolution, revealing genetic variations possibly linked to thermal adaptation.

Introduction

The rapid advancement of high-throughput sequencing has over the last decade revolutionized genomic research with the increasing numbers of whole genome resources available for multiple vertebrate species, including the diverse group of teleost fishes (Volff 2005; Ellegren 2014; Goodwin *et al.* 2016; Malmstrøm *et al.* 2016; 2017). However, whole genome sequencing (WGS) and generation of high quality genome assemblies are still considered costly and time-consuming (Jones and Good 2015). For investigations concerning specific genomic regions there is no need for complete genome information, which has spurred the development of reduction complexity approaches such as targeted sequence capture (Teer *et al.* 2010; Grover *et al.* 2012; Samorodnitsky *et al.* 2015). The basic idea of targeted sequence capture involves design of specific probes covering the particular genomic area of interest generating an enriched coverage of the targeted sequences (Turner *et al.* 2009; Grover *et al.* 2012). Most studies using targeted sequence capture have to a large extent been directed towards the exome, often supported by the existence of a reference genome (Ng *et al.* 2009; Broeckx *et al.* 2014; Yoshihara *et al.* 2016), or transcriptome assemblies (Syring *et al.* 2016). Recent reports have, however, been focusing on off-target sequences in noncoding regions (Guo *et al.* 2012; Samuels *et al.* 2013; Syring *et al.* 2016; Yoshihara *et al.* 2016; Morin *et al.* 2016), as they may contain crucial regulatory elements varying in sequence and length between populations or species, and could be of functional and evolutionary importance (Woolfe *et al.* 2004; Patrushev and Kovalenko 2014).

To our knowledge, sequence capture studies have so far been based on short-read sequencing technology (George *et al.* 2011; Samorodnitsky *et al.* 2015; Li *et al.* 2015; Bragg *et al.* 2016), and construction of continuous sequences enabling resolution of gene organization have therefore not been possible. Comparative genetic studies of gene organization or synteny requires longer, more continuous stretches of DNA containing more than one gene (Huddleston *et al.* 2014). By its ability to span long stretches of repeats, long-read sequencing technology has been successfully applied to improve genome assembly statistics and generation of highly continuous genome assemblies for a growing number of species (English *et al.* 2012; Kim *et al.* 2014; Bickhart *et al.* 2017; Korlach *et al.* 2017; Tørresen *et al.* 2017a; Tørresen *et al.* 2017b). For example, incorporation of long PacBio reads resulted in a significantly improved version of the Atlantic cod (*Gadus morhua*) genome assembly, i.e. a 50-fold increase in sequence continuity and a 15-fold reduction in the proportion of gaps (Tørresen *et al.* 2017b). Correspondingly, utilizing long-read sequencing technology in combination

with targeted capture could yield longer continuous assemblies of specific genomic regions of interest, allowing in-depth comparative genetic studies, even in species where a reference genome is not available.

In fishes, the hemoglobin (*Hb*) gene family, encoding the protein subunits Hba and Hbb, has shown to be of importance for ecological adaptation, as environmental factors such as temperature directly influences the ability of Hb to bind O_2 at respiratory surfaces and its subsequent release to tissues (Wells 2005). In a recent report, a full characterization of the *Hb* gene repertoire – using comparative genomics analysis – uncovered a remarkably high *Hb* gene copy variation within the codfishes (Baalsrud *et al.* 2017). Notably, a negative correlation between the number of *Hb* genes and depth of which the species occur was observed, suggesting that the more variable environment in epipelagic waters have facilitated a larger and more diverse *Hb* gene repertoire, which was supported by evidence of diversifying selection (Baalsrud *et al.* 2017). Furthermore, in Atlantic cod, two tightly linked polymorphisms at amino acid positions 55 and 62 of the Hbb1-globin – suggested to be associated with thermal adaptation – exhibit a latitudinal cline in allele frequency in populations inhabiting varying temperature and oxygen regimes in the North Atlantic and Baltic Sea (Andersen *et al.* 2009). Populations found in the southern regions display the Hbb1-1 variant (Met55Lys62), whereas more northern populations largely display the Hbb1-2 variant (Val55Ala62) (Andersen *et al.* 2009). The Hbb1-1 variant has been shown to be insensitive to temperature whereas Hbb1-2 is temperature dependent with a higher O_2 affinity than Hbb1-1 at colder temperatures (Karpov and Novikov 1980; Andersen *et al.* 2009), however, this has been questioned by (Barlow *et al.* 2017). Additionally, an indel polymorphism within the promoter of the *Hbb1* gene has been reported to be in linkage disequilibrium with the above-mentioned polymorphisms (Star *et al.* 2011). Examination of multiple Atlantic cod populations uncovered that a longer promoter variant is associated with *Hbb1-2* and found to up-regulate its gene expression at higher temperatures, i.e. aiding in the maintenance of total oxygen-carrying capacity (Star *et al.* 2011).

In teleosts, the *Hb* genes are found to reside at two distinct genomic regions – the MN and LA cluster. Earlier reports have shown that there is a high evolutionary turnover of *Hb* genes across teleosts, with lineage-specific duplications and losses, which is in stark contrast to genes flanking the *Hb* genes, where the synteny is highly conserved (Quinn *et al.* 2010; Opazo *et al.* 2013; Feng *et al.* 2014). In this study, the overall goal was to elucidate the evolutionary past of the *Hb* clusters – including *Hb* genes, flanking genes and intergenic sequences – within the phylogenetically diverse group of codfishes (Gadiformes) by taking the advantage of long read sequencing

technology combined with targeted sequence capture. Eight codfish species were carefully selected on the basis of both phylogenetic and habitat divergence, implying that they are exposed to a variety of environmental factors as well as displaying distinct life-history traits. A highly continuous genome assembly of Atlantic cod (Tørresen *et al.* 2017b) as well as low coverage draft genome assemblies of all eight species (Malmstrøm *et al.* 2017) were used in the design of the probes covering the genomic regions of interest. To enable targeted sequence capture for PacBio RSII sequencing, we modified the standard protocol for sequence capture offered by NimbleGen, i.e. the SeqCap EZ (Roche NimbleGen), as well as generating custom-made barcodes. This combined approach resulted in successful capturing and assembling of the two *Hb* gene clusters across the codfishes examined. The generation of highly continuous assemblies – for most of the species – enabled reconstruction of micro-synteny revealing lineage-specific gene duplications and identification of a relatively large and inter-species variable indel located in the promoter region between the *Hbb1* and *Hba1* genes.

Our study demonstrates that this approach, combining sequence capture technology with long-read sequencing is a highly efficient and versatile method to investigate specific genomic regions of interest – with respect to micro-synteny, regulatory regions and genetic organization – across distantly related species where genome sequences are lacking.

143 # Results

144 ## Capture and *de novo* assembly of the target regions

145 The capture probe design (workflow schematically shown in Figure 1) resulted in a
146 total of 7057 probes based on the target region in Atlantic cod, covering 337 kbp of
147 sequence. 26774 probes were designed for the additional codfishes, covering in total
148 an area of 1.82 Mbp of target sequence. The target region and the *Hb* gene clusters
149 were successfully captured and enriched for eight codfishes; Atlantic cod (*Gadus*
150 *morhua*), haddock (*Melanogrammus aeglefinus)*, silvery pout (*Gadiculus argenteus*), cusk
151 (*Brosme brosme*), burbot (*Lota lota*), European hake (*Merluccius merluccius*), marbled
152 moray cod (*Muraenolepus marmoratus*), and roughhead grenadier (*Macrourus berglax*),
153 with number of reads spanning from 35573 to 73005 (Table 1). The average read
154 length was 3032 bp, varying from 2836 bp in European hake to 3265 bp in burbot,
155 resulting in the capture of an average of 16.71 Mbp per species (Table 1). By mapping
156 reads back to the capture target region we found that the average mapping depth
157 was variable across the target region for all species (Figure 2 and 3). Because of the
158 skewed distribution of mapping depth, we also calculated median depth, which was,
159 as expected, the highest for Atlantic cod at 242x (Table S1). The median mapping
160 depth was consistently high for most of the other species as well, with the lowest for
161 roughhead grenadier (12x). Both median and average depths for the MN region are
162 persistently higher than for the LA region for all species, with the exception of silvery
163 pout (Table S1). Furthermore, positions with high degree of mapping corresponded
164 to the location of the genes used in the design of the capture probes across all species
165 (Figure 2 and Figure 3). The percentage of reads mapping to the target region ranged
166 from 25-43%, however, the percentage of the target region covered by reads ranged
167 from 53-100% with five species having more than 90% of the target region covered by
168 reads (Figure 4c and Table S1).

169 To address factors influencing capture success we compared various capture
170 statistics to overall genomic divergence between the Atlantic cod genome and
171 independent WGS data for each species from (Malmstrøm *et al.* 2017) (Table S1). We
172 found a strong negative correlation between genomic divergence to Atlantic cod and
173 median mapping depth against the target region (r=-0.90, Figure 4a), percent of reads
174 mapped to the target region (r=-0.90, Figure 4b), and percentage of reads mapped to
175 the target region (r=-0.84, Figure 4c).

176 We constructed *de novo* assemblies with quite consistent assembly statistics
177 across species. Contig N50 ranged from 8055 bp in burbot to 6523 bp in European
178 hake and the total number of contigs varied from 205 in burbot to 455 in marbled

179 moray cod. However, there was some variation in the size of the largest contig,
180 which ranged from 79 kbp in Atlantic cod to 30 kbp in marbled moray cod (Table 1).
181 To evaluate whether the assemblies represent the actual target regions we mapped
182 the *de novo* assemblies for each species to the target region in Atlantic cod, for which
183 the capture design is largely based upon (Figure 2 and 3). As expected, the
184 assemblies corresponded to the regions with high coverage of reads, i.e. the areas of
185 the target region containing genes included in the probe design.

186 Synteny of the *Hb* gene regions

187 Our capture design combined with long-read PacBio sequencing allowed us to
188 reconstruct micro-synteny of the MN and LA regions for Atlantic cod, haddock,
189 silvery pout, cusk, burbot, European hake, marbled moray cod and roughhead
190 grenadier (Figure 5). From the *de novo* assemblies, we were able to identify the
191 majority of the *Hb* genes and all of the flanking genes, which show that our capture
192 design was successful. However, the degree of continuity varied in the different
193 assemblies. In Atlantic cod, haddock, silvery pout, cusk, burbot and European hake
194 we could infer micro-synteny revealing that *Hb* and their flanking genes organization
195 largely followed what has previously been reported for Atlantic cod (Figure 5) (Star
196 *et al.* 2011). We found *Hbb4* only to be present in Atlantic cod (Figure 5b), which is in
197 line with (Baalsrud *et al.* 2017). Furthermore, the *de novo* assemblies confirmed a
198 linage-specific duplication of *Hbb2* in the roughhead grenadier (Baalsrud *et al.* 2017).
199 Additionally, we identified a complete *Hba4*-like gene in the assembly of the marbled
200 moray cod, not earlier identified in this species. However, the *Hba4*-like gene in
201 marbled moray cod is likely a pseudogene due to a frameshift mutation causing
202 multiple stop codons. Furthermore, we were able to identify most of the *Hb* genes
203 reported in the recent study of (Baalsrud *et al.* 2017), however, a few are missing from
204 our dataset (Figure 5a and b). Pairwise sequence alignment of these paralogous *Hb*
205 genes from (Baalsrud *et al.* 2017) revealed sequence identities up to 98 % (Table S2).

206 Target region in the haddock and Atlantic cod genome assemblies

207 As a proof of concept, we reconstructed synteny of the target region in the most
208 recent genome assemblies of Atlantic cod (gadMor2 (Tørresen *et al.* 2017b)) and
209 haddock (melAeg (Tørresen *et al.* 2017a)). In Atlantic cod, the MN region is located
210 on linkage group 2 (Figure 5a) and LA on linkage group 18 (Figure 5b), in haddock
211 MN is located on scaffold MeA_20160214_scaffold_771 (Figure 5a) and LA on
212 scaffold MeA_20160214_scaffold_1676 (Figure 5b). The overall synteny in Atlantic
213 cod was congruent with (Wetten *et al.* 2010) except for the relative direction of the
214 genes *foxj1a* and *rhbdf1.* Furthermore, the organization of *Hb*s and their flanking

genes in the genome assembly of haddock is conserved compared to Atlantic cod with the exception of *Hbb4* in the MN region, which is absent in haddock (Figure 5).

Repetitive sequences in the in the *Hb* gene regions

Quantifying the amount of repetitive sequences in the target region(s) was only possible for Atlantic cod (gadMor2) and haddock (melAeg), for which high-quality genome assemblies exist. The amount of repetitive sequences in the target region differed between the MN cluster and the LA cluster in Atlantic cod. The MN region (214 kb) contained a total of 10.7% repeated sequences, including 1.0% retro-elements, 1.3% transposons, 5.8% simple repeats, and 2.6% of various low complexity and unclassified repeated sequences (Table S3). In comparison, in the smaller LA region (123 kb) the proportion of repeated sequences was twice as high (20.3%), which comprised of 2.8% retro-elements, 2.4% transposons, 13.8% simple repeats, and 1.3% of various low complexity and unclassified repeated sequences. Furthermore, the orthologous target regions in haddock followed the same pattern. The MN region contained 16.3 % repeated sequences, in contrast to 19.8 % found in the LA region (Table S3).

Insertions and deletions in the promoter region of *Hba1 – Hbb1*

The previously shown 73 bp indel in the bi-directional promoter region of *Hba1* and *Hbb1* – discerning the cold-adapted migratory Northeast Artic cod (NEAC) from the more temperate-adapted southern Norwegian coastal cod (NCC) (Star *et al.* 2011) – was confirmed by the improved version of the NEAC assembly (gadMor2). The continuity of our capture assemblies (Figure 5) enabled location of the orthologous captured regions in haddock, silvery pout and cusk. In each of the species an indel of variable length were identified (Figure 6). Compared to the long promoter variant – found to be linked with the *Hbb1-2* in Atlantic cod – the indel is shorter in the other species by 11 bp in haddock, 22 bp in silvery pout and 56 bp in cusk (Figure 6). Although the indels are varying in length, the conserved flanking sequences in the alignment clearly show that they represent orthologous regions. Moreover, we found the amino acid positions at 55 and 62 of the *Hbb1* gene to vary between species; Haddock has Val55-Lys62, silvery pout has Met55-Gln62, while cusk has Met55-Lys62 similarly to NEAC (Figure 6). Additionally, we investigated amino acid positions 55 and 62 in the *Hbb1* gene across a number additional codfish species for which we have available gene sequences from (Baalsrud *et al.* 2017), revealing these sites to be variable across this lineage (Table S4). Ancestral reconstruction of *Hbb1* demonstrated that the ancestral state in position 55 was Met in codfishes, and in

250 position 62 was Lys in all codfishes except *Bregmaceros cantori* (Supplementary
251 Figures S1 and S2).

Discussion

Capture of *Hb* gene clusters with 70 million years divergence time reveal conserved synteny and lineage-specific *Hb* duplications

We here demonstrate a successful in-solution targeted sequence capture and assembling of coding and noncoding sequences of the *Hb* clusters from codfish species separated by up to 70 million years (My) of evolution. Two features make our approach unique from earlier studies. First, the target regions consisted of both coding and noncoding genomic sequences. Second, we designed capture of large fragments – combined with development of custom-made probes – in order to utilize the long-read PacBio sequencing platform. This is in contrast to current targeted capture sequencing protocols that are based on short-read sequencing technologies (George *et al.* 2011; Mascher *et al.* 2013).

The organization and orientation of the *Hb* flanking genes that we identified were conserved across all species (Figure 5a and b). However, in concordance with earlier studies of the *Hb* region, we found significant variation in copy numbers of the *Hb* genes, with linage specific duplications and losses (Star *et al.* 2011; Opazo *et al.* 2013; Feng *et al.* 2014; Baalsrud *et al.* 2017). We only found *Hbb4* in Atlantic cod, supporting earlier studies showing that *Hbb4* is the result of a recent duplication in this species (Borza *et al.* 2009; Baalsrud *et al.* 2017). Interestingly, the presence of two copies of *Hbb2* on the same contig in the roughhead grenadier *de novo* assembly confirmed a lineage specific gene duplication of *Hbb2*, which was found in a recent study of *Hb*s in codfishes (Baalsrud *et al.* 2017). Additionally, a copy of the *Hba4* was found in the *de novo* assembly of the marbled moray cod not found in (Baalsrud *et al.* 2017). The presence of a frame-shifting mutation that is causing multiple stop codons indicated that this *Hba4* gene is most likely a pseudogene. *Hba4* is also a pseudogene in the closely related species *Mora moro*, *Trachyrincus scabrus*, *T. murrayi* and *Melanonus zugmayeri* (Baalsrud *et al.* 2017). Although we identified most of the *Hb* genes from (Baalsrud *et al.* 2017), a few were absent from this dataset (Figure 5a and b), which we suspect may be due to collapse of paralogous *Hb* genes, as they may have as high as 98% sequence identity (Table S2).

Length variation in the bi-directional *Hba1-Hbb1* promoter within the codfishes

The discovery of a promoter of variable length between *Hba1* and *Hbb1* in different species (Figure 6) was concordant with earlier findings of length variation in the homologous region in different populations of Atlantic cod (Star *et al.* 2011). The

287 migratory NEAC population has been shown to harbor the 73 bp longer variant at a
288 higher frequency compared to coastal cod populations (see Figure 6 and (Star *et al.*
289 2011)). Interestingly, we found relatively long promoters with high sequence
290 similarity to the NEAC indel in haddock and silvery pout. In contrast, cusk
291 displayed a relatively short promoter, however, still 17 bp longer than in NCC
292 (Figure 6). Furthermore, we found the amino acid positions 55 and 62 in *Hbb1,*
293 known to be polymorphic in Atlantic cod, to be variable across all codfishes included
294 in this study (Figure 6). Investigations of the same positions in a number additional
295 codfishes for which we have available gene sequences (Baalsrud *et al.* 2017), revealed
296 that these positions are highly variable across this linage (Table S2). Notably, the
297 most likely ancestral state of codfish *Hbb1* is Met55Lys62 (Supplementary Figures S1
298 and S2). Cusk and the coastal/southern Atlantic cod thus both display the ancestral
299 state as well as a short promoter, although the cusk promoter was 17 bp longer
300 (Figure 6). Collectively, these results suggest two different scenarios for promoter
301 length evolution. Scenario 1: The short promoter represents the ancestral state of the
302 Gadidae-family (including cusk and Atlantic cod; see (Malmstrøm *et al.* 2016)) and
303 that silvery pout and some populations of Atlantic cod have evolved a longer
304 promoter. Scenario 2: The long promoter is the ancestral state with independent
305 deletions of variable lengths in cusk, silvery pout, haddock and costal/southern
306 Atlantic cod (*Hbb1-1*). To disentangle this, we would need to obtain promoter
307 sequences from additional gadiform species. Regardless, the short-long promoter
308 polymorphism has been maintained throughout speciation events based on the
309 presence of both variants in Atlantic cod. Moreover, in both scenarios, cusk and
310 Atlantic cod (*Hbb1-1*) have maintained the ancestral Met55Lys62, while silvery pout,
311 haddock and Atlantic cod (*Hbb1-2*) have acquired substitutions at these positions due
312 to similar selection pressures or genetic drift. In this regard, it could be mentioned
313 that the NEAC, haddock and silvery pout display migratory behavior (e.g. diurnally
314 feeding movements as well as seasonal spawning migrations) compared to the more
315 stationary cusk and coastal cod (Eschemeyer and Fricke 2017) which could mean that
316 they have a higher O_2 demand and are exposed to greater temperature variation,
317 which in turn has selected for a temperature-dependent long promoter. Furthermore,
318 given that promoter length and positions 55/62 at *Hbb1* are important genetic
319 components of temperature adaptation in Atlantic cod populations (Star *et al.* 2011),
320 they most likely play a role in temperature adaptation in the other codfishes.

321 **Assembly success affected by probe design and repeat content**

322 In some species, nearly the complete target region is assembled in large contigs
323 containing multiple genes including cusk, whereas in other species such as the more

324 distantly related roughhead grenadier, the cluster is more fragmented (Figure 5). In
325 all species, the areas of the target regions that harbor genes of which probes are
326 designed for, as well as any areas containing repeated sequences, have very high
327 depths in comparison to the areas of intergenic sequences (Figure 2 and 3). This
328 poses a challenge for the assembly software, which is based in the assumption of
329 uniform depth over the sequencing data (Miller *et al.* 2010).
330 Overall, the MN cluster seems to be more successfully assembled than the LA
331 cluster, which is more fragmented (Figure 5). Differences in assembly completeness
332 between the two regions might be a result of several factors. Firstly, the MN region
333 has more flanking genes in closer proximity to the *Hb* region, which results in a
334 higher density of probes. Secondly, the overall repeat content of the LA region is one
335 order of magnitude larger than in the MN region, largely due to the larger
336 proportion of simple repeats. Repeat content is a major interference in capture
337 experiments because unwanted repetitive DNA may be enriched for, especially if
338 there are repeated sequences included in the probe design. Furthermore, if probes
339 were not completely covered by target DNA they get single-stranded sticky ends that
340 can hybridize to repetitive or other non-target DNA (Newman and Austin 2016).
341 Lastly, unless there were some longer reads that bridged such areas, this would in
342 turn have led to gaps in the downstream *de novo* assemblies. Following that the
343 assembly success was possibly a result of read length, we reason that a future
344 increase of the average read length from 3 kbp to 5-10 kbp, would be sufficient to
345 substantially increase the completeness of the assemblies. Due to the current circular
346 consensus (CCS) PacBio sequencing technology, however, which is a trade-off
347 between accuracy and length of reads, longer reads with sufficient accuracy are not
348 feasible.

349 Long-read sequencing capture across species harbors new potential for 350 comparative genomic studies

351 The number of reads mapping to the target region was in the range of 23-43%, which
352 may seem low compared with other capture studies. For instance, a whole exome
353 capture study on humans reported 56.1% of reads mapped to the target region (Guo
354 *et al.* 2012) and a similar study in rats reported to have 78.3% of reads on target
355 (Yoshihara *et al.* 2016). In contrast to our study however, these capture experiments
356 enriched either the exome or ultra-conserved elements within a single species.
357 Furthermore, we were able to cover up to 98% of the target region with >10 reads
358 across species (Table S1) which is similar to what mentioned experiments within
359 human and rat exomes reported (Guo *et al.* 2012; Yoshihara *et al.* 2016) and the main
360 difference is the higher percentage of non-target sequences in our study.

361 We were able to capture complete genes for species with 70 My divergence
362 time from the Atlantic cod (Figure 5). As expected, we found that capture success
363 declines with increased sequence divergence between the reference genome of which
364 we chiefly based our capture probes and the genomes of the included codfishes
365 (Figure 4). It has been reported that orthologous exons were successfully captured in
366 highly divergent frog species (with 200 My of separation), nevertheless the capture
367 success greatly decreased with increased evolutionary distance (Hedtke *et al.* 2013).
368 Similarly, it has been demonstrated that it is possible to capture >97% of orthologous
369 sequences in four species of primates that diverged from humans 40 My ago, using
370 probes entirely based on the human exome (George *et al.* 2011). Further, exomes were
371 effectively captured from skink species that diverged up to 80 My from the reference,
372 yet reporting a substantial decline in capture efficiency for sequences >10 % different
373 from the reference species (Bragg *et al.* 2016). Our study stands out from previous
374 capture experiments because intergenic, noncoding sequences in addition to genes
375 were captured. Efficient capture of intergenic sequences requires less divergence
376 time, as these regions usually evolve faster than genes (Koonin and Wolf 2010). Thus,
377 the most distantly related species from Atlantic cod for which we captured both
378 coding and noncoding sequences was burbot, which diverged from Atlantic cod 46
379 My (Figure 5). We argue, in line with (Schott *et al.* 2017), that sequence divergence
380 may be a more exact predictor of capture success than evolutionary distance, as the
381 sequence capture process is mainly influenced by the difference between the probe
382 sequence and the target sequence. European hake, marbled moray cod and
383 roughhead grenadier all diverged from cod about 70 My ago, however, the European
384 hake *Hb* regions was more successfully captured and assembled (Table 1; Figure 2).
385 This could be due to European hake having a lower genome-wide divergence to
386 Atlantic cod than marbled moray cod and roughhead grenadier (809k vs 879k and
387 907k SNPs; Table S1).
388 Finally, it should be mentioned that cusk – which diverged from Atlantic cod
389 39 My ago – was added to the experimental design after the species-specific probes
390 were generated. Thus, the successful capture of cusk was therefore solely based on
391 cross-species target enrichment, and could most likely been further improved if
392 species-specific probes for this species have been included.

393 Concluding remarks and future perspectives

394 Here, we have successfully demonstrated that combining targeted sequence capture
395 with long-read sequencing technology is as an efficient approach to obtain high
396 quality sequence data of a specific genomic region, including both coding and

397 noncoding sequences, across evolutionary distant species. We show that genome-
398 wide divergence is of importance for capture success across species. Furthermore, the
399 use of long-read sequencing augmented the *de novo* assembly of regions containing
400 repeated sequences that would otherwise fragment assemblies based on short-read
401 sequencing. This is crucial for capturing complete intergenic sequences that may be
402 highly divergent compared to genic regions even among fairly closely related
403 species. Given the rapid development in sequencing technologies future methods
404 will enable read-through of repeated regions and thus further increase the
405 completeness of assemblies. Moreover, a less stringent hybridization protocol should
406 make it possible to capture sequences across even deeper evolutionary time. In sum,
407 our approach has the potential of enhancing comparative genomic studies of
408 continuous genic and intergenic regions between any eukaryotic species-group
409 where genomic resources are scarce.

410 # Material and methods

411 ## Defining target region and probe design

412 The probe design was chiefly based on the high-quality genome of Atlantic cod,
413 known as gadMor2 (Tørresen *et al.* 2017b). In addition, species-specific probes were
414 designed based on low-coverage assembled genomes (Malmstrøm *et al.* 2016) for ten
415 selected species representing six families in the Gadiformes order. These species
416 were Atlantic cod (*Gadus morhua*), Alaskan Pollock (*Gadus chalcogrammus*), polar cod
417 (*Boreogadus saida*), haddock (*Melanogrammus aeglefinus*), Silvery pout (*Gadiculus*
418 *argenteus*), burbot (*Lota lota*), European hake (*Merluccius merluccius*), roughhead
419 grenadier (*Macrourus berglax*), roughsnout grenadier (*Trachyrincus scabrus*) and
420 marbled moray cod (*Muraenolepus marmoratus*).
421 To retrieve relevant sequence data for the probe design, the MN and LA *Hb*
422 regions were extracted from gadMor2 (Figure 1). These sequences, hereby known as
423 the target region, were then used queries in BLAST (Altschul *et al.* 1990) searches
424 with an E-value threshold of <0.1 against the genome assembly data of all ten species.
425 In total, 5604 sequences from the chosen species were supplied to NimbleGen
426 probe design. Protein coding genes from the ENSEMBL database were used to define
427 the regions to be tiled in the probe design (Table S5) within the target region of the
428 Atlantic cod, and the unitigs for each of the additional codfishes.
429 NimbleGen SeqCap EZ capture probes were designed by NimbleGen (Roche,
430 Madison, USA) using a proprietary design algorithm. NimbleGen offers an in-
431 solution sequence capture protocol, which includes custom made probes. Uniquely,
432 the capture probes from NimbleGen are tiled to overlap the target area. 50 – 100 bp
433 (average 75 bp) probes where designed tiled over the target region (subset of
434 gadMor2) resulting in each base, on average, being covered by two probes.
435 Additionally, raw reads from Illumina sequencing from (Malmstrøm *et al.* 2017) were
436 used for each species to estimate repetitive sequences in each of the species'
437 genomes, aiming to discard probes containing any repeats.

438 ## Sample collection and DNA extraction

439 Our goal working with animals is always to limit any harmful effects of our research
440 on populations and individuals. Whenever possible we try to avoid animals being
441 euthanized to serve our scientific purpose alone by collaborating with commercial
442 fisheries or museums. The tissue samples used in this study are either from
443 commercially fished individuals intended for human consumption or museum
444 specimen. The commercially caught fish were immediately stunned, by bleeding

445 following standard procedures by a local fisherman. There is no specific legislation
446 applicable to this manner of sampling in Norway, however it is in accordance with
447 the guidelines set by the 'Norwegian consensus platform for replacement, reduction
448 and refinement of animal experiments' (www.norecopa.no).
449 DNA was extracted from tissue samples using High Salt DNA Extraction
450 method by Phill Watts (https://www.liverpool.ac.uk/~kempsj/IsolationofDNA.pdf,
451 last day accessed: December 2017). The concentration and purity of the DNA samples
452 were quantified using NanoDrop (Thermo Scientific, Thermo Fisher Scientific,
453 Waltham, MA, USA) and a Qubit fluorometer (Invitrogen, Thermo Fisher Scientific,
454 Waltham, MA, USA). Due to poor DNA quality, three species included in the probe
455 design; Alaskan Pollock, polar cod and roughsnout grenadier were excluded from
456 further analysis. In total, eight species were sequenced; seven of these species were
457 included in the probe design and one closely related species (cusk, *Brosme brosme*),
458 which serves as a cross species capture experiment without species-specific probes.

459 **Capture, library preparation and sequencing**

460 The sequencing libraries were prepared following a modified Pacific Biosciences
461 SeqCap EZ protocol. As multiplexing of the samples before capture was required,
462 barcodes were designed at the Norwegian Sequencing Centre
463 (http://www.sequencing.uio.no) using guidelines from Pacific Biosciences
464 (Supplementary Materials and methods). Genomic DNA was sheared to 5 kb
465 fragments using MegaRuptor (Diagenode, Seraing (Ougrée), Belgium). Due to poorer
466 DNA quality, fragmenting was not done for European hake. For this sample together
467 with fragmented DNA from roughhead grenadier, short fragments were removed
468 using BluePippin (Sage Science, Beverly, MA, USA) before library preparation.
469 Illumina libraries were prepared using KAPA Hyper Prep kit (Kapa Biosystems,
470 Wilmington, MA, USA) and barcoded using different Illumina barcodes. PacBio
471 barcodes were implemented during pre-capture amplification of libraries. After
472 amplification, fragment length distribution was evaluated using Bioanalyzer (Agilent
473 Technologies, Santa Clara, CA, USA) and samples were pooled in equimolar ratio.
474 During hybridization, SeqCap EZ Developer Reagent (universal repeat blocker for
475 use on vertebrate genomes) and oligos corresponding to Illumina and PacBio
476 barcodes were used for blocking. Captured gDNA was amplified to ensure that
477 sufficient amount of DNA was available for PacBio library preparation. Size selection
478 of the libraries was performed using BluePippin. Final libraries were quality checked
479 using Bioanalyzer and Qubit fluorometer (Invitrogen, Thermo Fisher Scientific,
480 Waltham, MA, USA) and sequenced on RS II instrument (PacBio, Menlo Park, CA,

481 USA) using P6-C4 chemistry with 360 minutes movie time. In total, 9 SMRT cells
482 were used for sequencing.

483 ## *De novo* assemblies

484 Reads were filtered and de-multiplexed using the 'RS_reads of insert.1' pipeline on
485 SMRT Portal (SMRT Analysis version smrtanalysis_2.3.0.140936.p2.144836). Each set
486 of reads corresponding to a given species was crossed-checked with their respective
487 six-nucleotide Illumina adapter. Reads containing an incorrect Illumina adapter were
488 removed. Adapter sequences were then trimmed using the application Prinseq-lite
489 v0.20.4 (Schmieder and Edwards 2011). The trimmed reads were assembled *de novo*
490 using Canu v1.4 +155 changes (r8150 c0a988b6a106c27c6f993dfe586d2336282336a6)
491 (Berlin *et al.* 2015). The Canu software is optimized for assembling single molecule
492 high noise sequence data. We specified genome size as the size of the target region
493 (300 kbp). Additionally, we ran PBJelly (English *et al.* 2012) on the Canu *de novo*
494 assemblies, using the raw reads to possible bridge gaps between scaffolds, settings
495 given in Supplementary Materials and Methods.
496 We assessed the assemblies by running Assemblathon 2 (Bradnam *et al.* 2013),
497 which reports assembly metrics such as the longest contig, the number of contigs,
498 and the N50 value. *De novo* assemblies of the MN and LA regions of Atlantic cod and
499 haddock were aligned and compared to their reference genomes, gadMor2 and
500 melAeg respectively, using BLAST and BWA v0.7.10 (Li and Durbin 2009) to
501 determine syntenic similarities and assembly completeness.

502 ## Estimating capture success

503 PacBio reads for all the species were mapped back to the Atlantic cod genome
504 assembly (gadMor2) in order to determine sequence capture success and target
505 mapping depths. Mapping was done using BWA-MEM v0.7.10 (Li and Durbin 2009).
506 Target-area read depth for all the species based on mapping against gadMor2, were
507 calculated using Samtools v1.3.1 (Li *et al.* 2009). We calculated both average and
508 median mapping depth against the target region as a whole and for the MN and LA
509 region separately. We also calculated percentage of reads that mapped to the target
510 region, and the percentage of the target regions covered by reads to a minimum
511 depth of 10x. To compare assemblies to the target region we additionally mapped the
512 assemblies to the target region. In order to verify the sequence capture process,
513 sequence data for Atlantic cod and haddock were mapped back to their reference
514 genomes using BWA-mem v0.7.10 (Li and Durbin 2009). The results were visualized
515 using Integrative Genome Viewer (Robinson *et al.* 2011).

516 To obtain an independent measure of divergence between species in the
517 capture experiment we calculated genome wide level of divergence of each species to
518 the reference genome of Atlantic cod using low-coverage whole-genome sequence
519 data from (Malmstrøm *et al.* 2017). We mapped raw reads to Atlantic cod using
520 BWA-MEM (Li and Durbin 2009) and called SNPs using the Freebayes variant caller
521 (Garrison and Marth 2017). Some species are more closely related to Atlantic cod
522 than others, which could introduce a bias in mapping. To avoid this, we only looked
523 at genomic regions where all species mapped. The number of SNPs was then used as
524 an estimate of genome-wide divergence of each species to Atlantic cod. We also
525 mapped a low-coverage genome of Atlantic cod to the Atlantic cod reference genome
526 as a control.
527 In pursuance of factors explaining capture success we tested for correlations
528 and plotted the relationship between the genome wide level of divergence and the
529 following variables; median mapping depth against the target region (for total, LA
530 and MN, respectively); percentage of reads that mapped to the target region; and the
531 percentage of the target region covered by reads. All tests and plots were done using
532 R version 3.2.5(Team 2013).
533 Assembly continuity is very often hampered by the presence of repeats, which
534 create gaps. We therefore quantified repeat-content in the target region extracted
535 from gadMor2 and orthologous regions in haddock using Repeatmasker Open 3.0
536 (Smit *et al.* 2010) for the MN region and the LA region separately.

537 **Identifying gene location and synteny**

538 In order to identify the genes of interest and their location in the assembly we used
539 local sequence alignment algorithm BLAST v2.4.0 (Altschul *et al.* 1990) with protein
540 sequences of the genes of interest (Table S5) as queries. tblastn was used with an e-
541 value of 0.1. Investigation of *Hbb1-Hba1* promoter region was done for four species,
542 Atlantic cod, haddock, silvery cod and cusk. Sequences were aligned with ClustalW
543 default settings using MEGA7 (Kumar *et al.* 2016). Ancestral sequence reconstruction
544 was carried out for *Hbb-1* gene sequences from 24 species of codfishes from (Baalsrud
545 *et al.* 2017) using a maximum likelihood method implemented in MEGA7 (Kumar *et*
546 *al.* 2016).
547 Additionally, we estimated sequence identity using EMBOSS Needle (Rice *et*
548 *al.* 2000) with default settings, between *Hbb* gene sequences from (Baalsrud *et al.*
549 2017) that where missing and present in the *de novo* assemblies to evaluate similarity
550 (Table S2).

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Author contributions

H.T.B. and S.J. initially conceived and designed the study, with input from S.N.K.H, A.T.-K., M.S., G.O., R.S., and K.S.J. Tissue samples were provided by S.J. and H.T.B. Probe design was carried out by T.R. with assistance from S.N.K.H and H.T.B. DNA extraction and sequence library preparation was performed by S.N.K.H and A.T.-K, respectively. Sequence capture was carried out by S.N.K.H, A.T.-K., M.S. and G.O. Filtering, mapping of sequences and *de novo* assemblies was done by S.N.K.H., assisted by O.K.T and H.T.B. Annotation of genes, synteny analyses, statistical analyses and construction of all figures and tables was done by S.N.K.H and H.T.B. The manuscript was written by S.N.K.H and H.T.B. with input from S.J. and K.S.J.

Competing interests

The authors declare that they have no competing interests.

Data and materials availability

All reads and assemblies (unitigs) reported on here, and the target region, subset of gadMor2 have been deposited at figshare under doi/xxx.

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Figure legends

Figure 1: Flowchart of sequence capture approach. a) Sequence data from the Atlantic cod genome (gadMor2 (Tørresen *et al.* 2017b)) combined with gene sequences of target genes and sequences from low coverage genomes of the additional codfishes are combined to generate probes. b) Isolated DNA is multiplexed with Illumina and PacBio barcodes. c) Raw reads for each species are used to score all probes, ensuring that no repeated sequences are present. DNA Probes are used in solution on isolated DNA for all of the included species, hybridizing to the target sequences. Target sequences are then captures and sequences on the PacBio RSII sequencing platform. d) Downstream bioinformatics includes de-multiplexing of reads and trimming, making the reads ready for downstream analysis such as mapping and *de novo* assembly.

Figure 2: Mapping of reads and assemblies against the MN target region. Each panel shows the reads and *de novo* assembly mapped against the MN target region in grey and orange, respectively, for species a.) Atlantic cod, b) haddock, c) silvery pout, d) cusk, e) burbot, f) European hake, g) marbled moray cod and h) roughhead grenadier. The positions of genes in the target region are indicated at the top.

Figure 3: Mapping of reads and assemblies against the LA target region. Each panel shows the reads and *de novo* assembly mapped against the LA target region in grey and orange, respectively, for species a) Atlantic cod, b) haddock, c) silvery pout, d) cusk, e) burbot, f) European hake, g) marbled moray cod and h) roughhead grenadier. The positions of genes in the target region are indicated at the top.

Figure 4: The relationship between capture success and genomic divergence to Atlantic cod. Linear regression of the relationship between the genomic divergence to Atlantic cod (SNPs x 10^5) and a) median mapping depth for the MN region (blue), LA region (red) and the combined target region (black); b) the percentage of reads mapping to the target region; c) the percentage of the target region covered by reads to a minimum depth of 10x. For each regression the correlation coefficient, r, is shown along with a p-value. Each data point is labeled by species according to this code: Ac=Atlantic cod, H=haddock, Sp=silvery pout, C=cusk, B=burbot, Eh=European hake, Mm=marbled moray cod and Rg=roughhead grenadier.

Figure 5: Synteny of the Hb gene clusters. Genomic synteny of the hemoglobin gene clusters shown at the top for the genomes of Atlantic cod (gadMor2 (Tørresen *et al.* 2017b)) and haddock (MelAeg (Tørresen *et al.* 2017a)). Below, the genomic synteny inferred from the *de novo* assemblies for all of the species included in the capture experiment. Stippled lines indicate assembly gaps – here we assume that the orientation of genes corresponds to the genomes of Atlantic cod and haddock. Gray boxes indicate genes that have been identified in (Baalsrud *et al.* 2017), but are absent

780 in the *de novo* assemblies. a) Synteny across the MN region b) Synteny across the LA
781 region.
782
783 **Figure 6: Polymorphisms in the bi-directional promoter between *Hba1* and *Hbb1***
784 **for five species in the Gadidae family.**
785 A schematic representation of *Hba1* and *Hbb1* with the promoter region between
786 them. The region contains an indel polymorphism of variable length across the five
787 species, as indicated by gaps. For each species/variant the alignment is shown along
788 with amino acid substitutions at positions 55 and 62 in the translated part of the *Hbb1*
789 gene.

Supporting Information Legends

Table S1: For each species, the average and median depth of reads mapped against the target region (for MN, LA and total), the genomic divergence to Atlantic cod (number of SNPs), percentage of nucleotides mapped to the target and the percentage of the target region with more than 10x coverage.

Table S2: Estimated sequence identity using EMBOSS Needle (Rice *et al.* 2000) with default settings, between paralogous Hbb gene sequences from (Baalsrud *et al.* 2017). Genes highlighted in bold are missing from the assemblies in figure 5.

Table S3: Amino acids at positions 55 and 62 in Hbb1 in various codfishes taken from (Baalsrud *et al.* 2017).

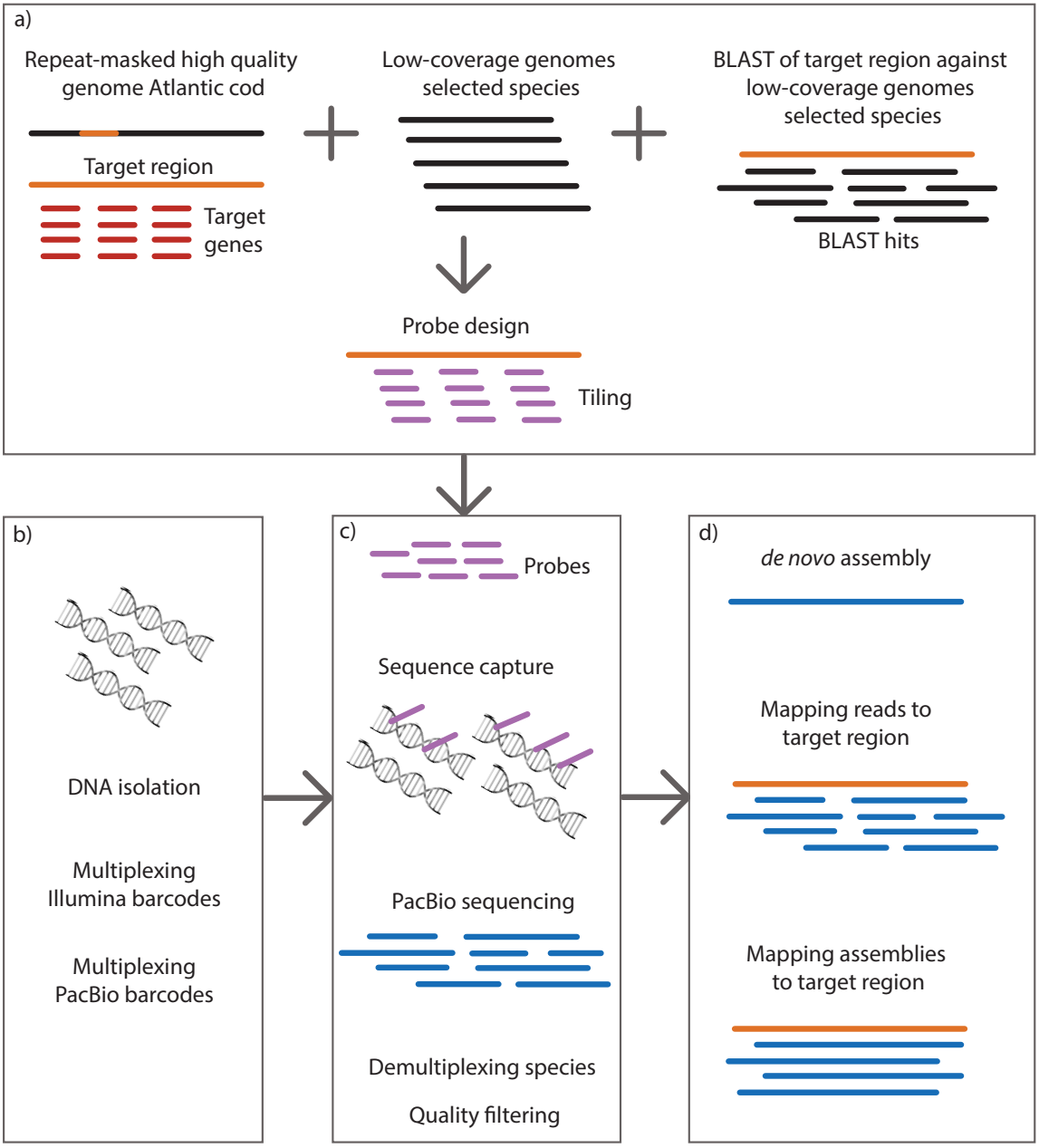
Table S4: Amount of repeated sequences in the target region of the Atlantic cod (gadMor2 (Tørresen *et al.* 2017b)) and haddock (melAeg (Tørresen *et al.* 2017a)) given in percentage.

Table S5: Genes provided Nimblegen for the probe design, and used to identify genes in *de novo* assemblies. For each gene, the gene name is given with its ENSEMBL name and ENSEMBL identifier.

Figure S1: Ancestral reconstruction of amino acids at position 55 in the *Hbb-1* gene in Gadiformes. Phylogenetic trees and ancestral reconstruction was carried out in MEGA 7.0.

Figure S2: Ancestral reconstruction of amino acids at position 62 in the *Hbb-1* gene in Gadiformes. Phylogenetic trees and ancestral reconstruction was carried out in MEGA 7.0.

818 **Figures**



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820 Figure 1
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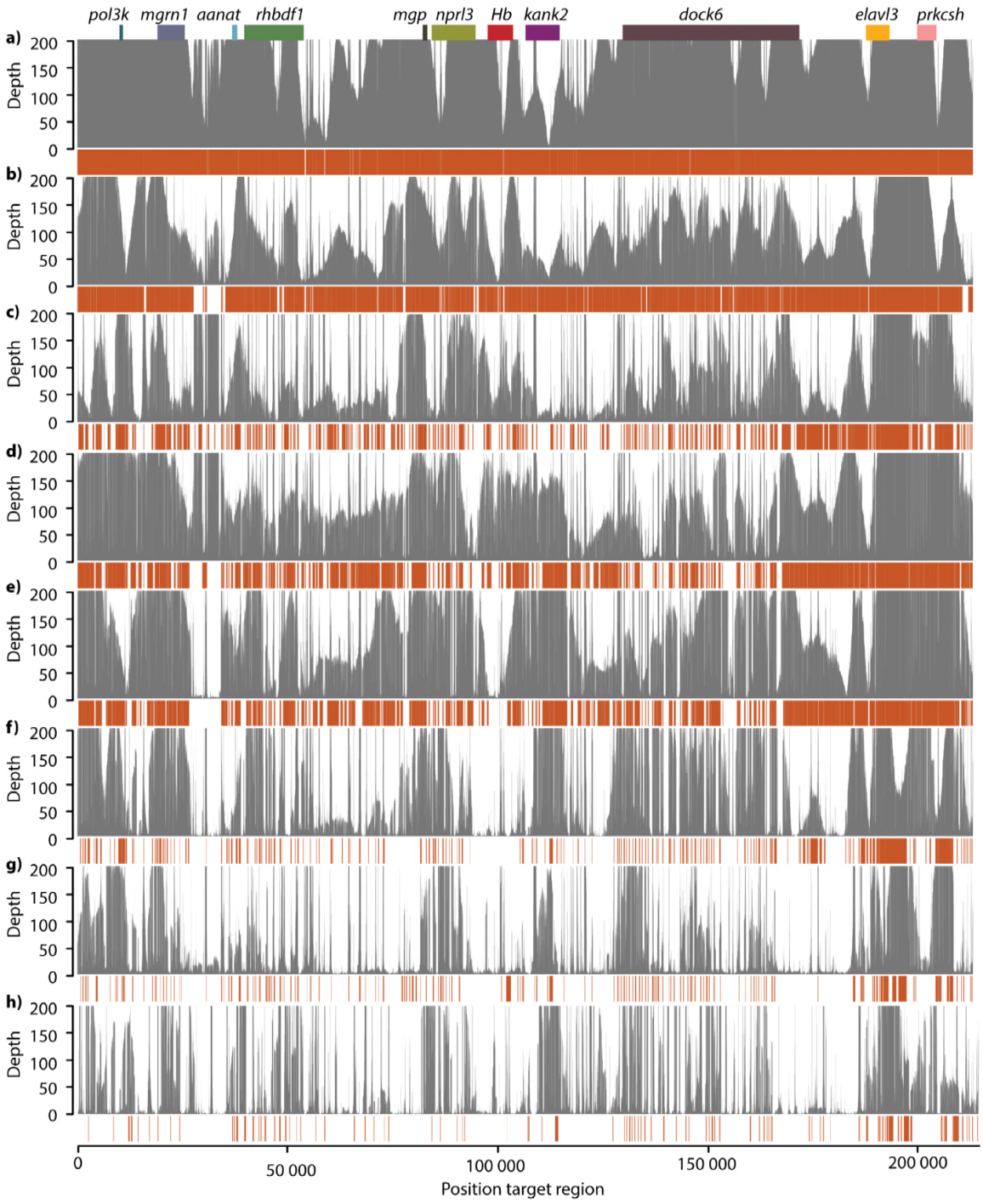
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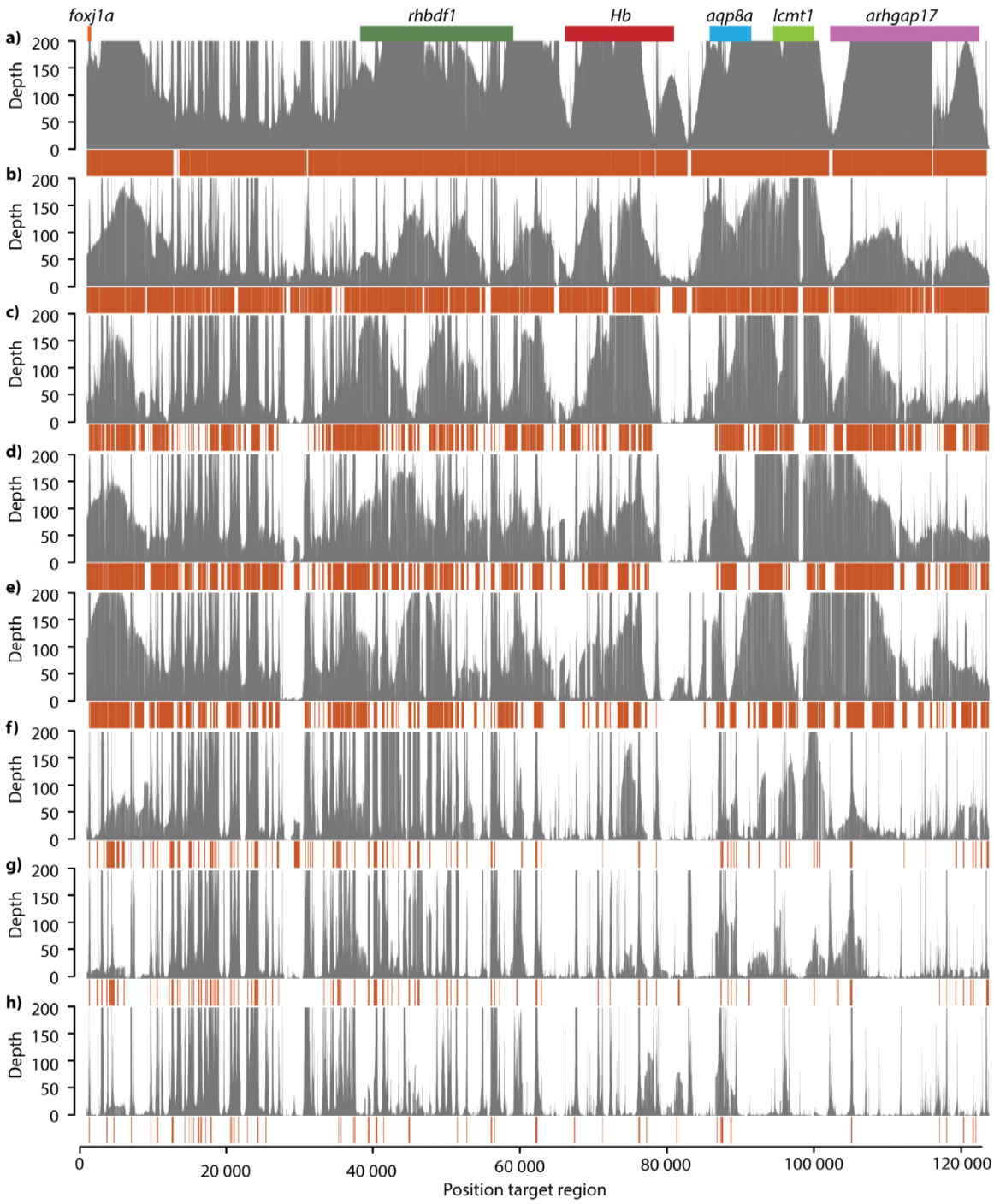
Figure 2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80 000
100 000
120 000
Position target region

Figure 3

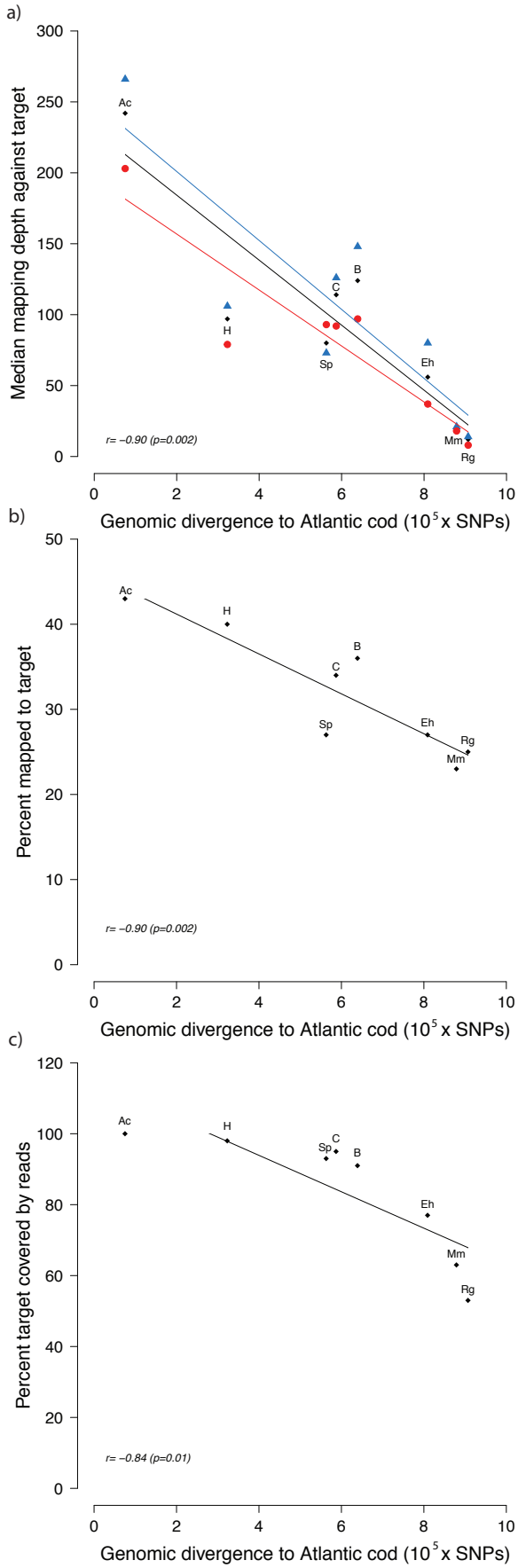
a)
Median mapping depth against target
300
250
200
150
100
50
0
Ac
H
C
B
Sp
Eh
Mm
Rg
r= −0.90 (p=0.002)
0
2
4
6
8
10
Genomic divergence to Atlantic cod (10^5 x SNPs)
b)
Percent mapped to target
50
40
30
20
10
0
Ac
H
B
C
Sp
Eh
Rg
Mm
r= −0.90 (p=0.002)
0
2
4
6
8
10
Genomic divergence to Atlantic cod (10^5 x SNPs)
c)
Percent target covered by reads
120
100
80
60
40
20
0
Ac
H
Sp
C
B
Eh
Mm
Rg
r= −0.84 (p=0.01)
0
2
4
6
8
10
Genomic divergence to Atlantic cod (10^5 x SNPs)

Figure 4

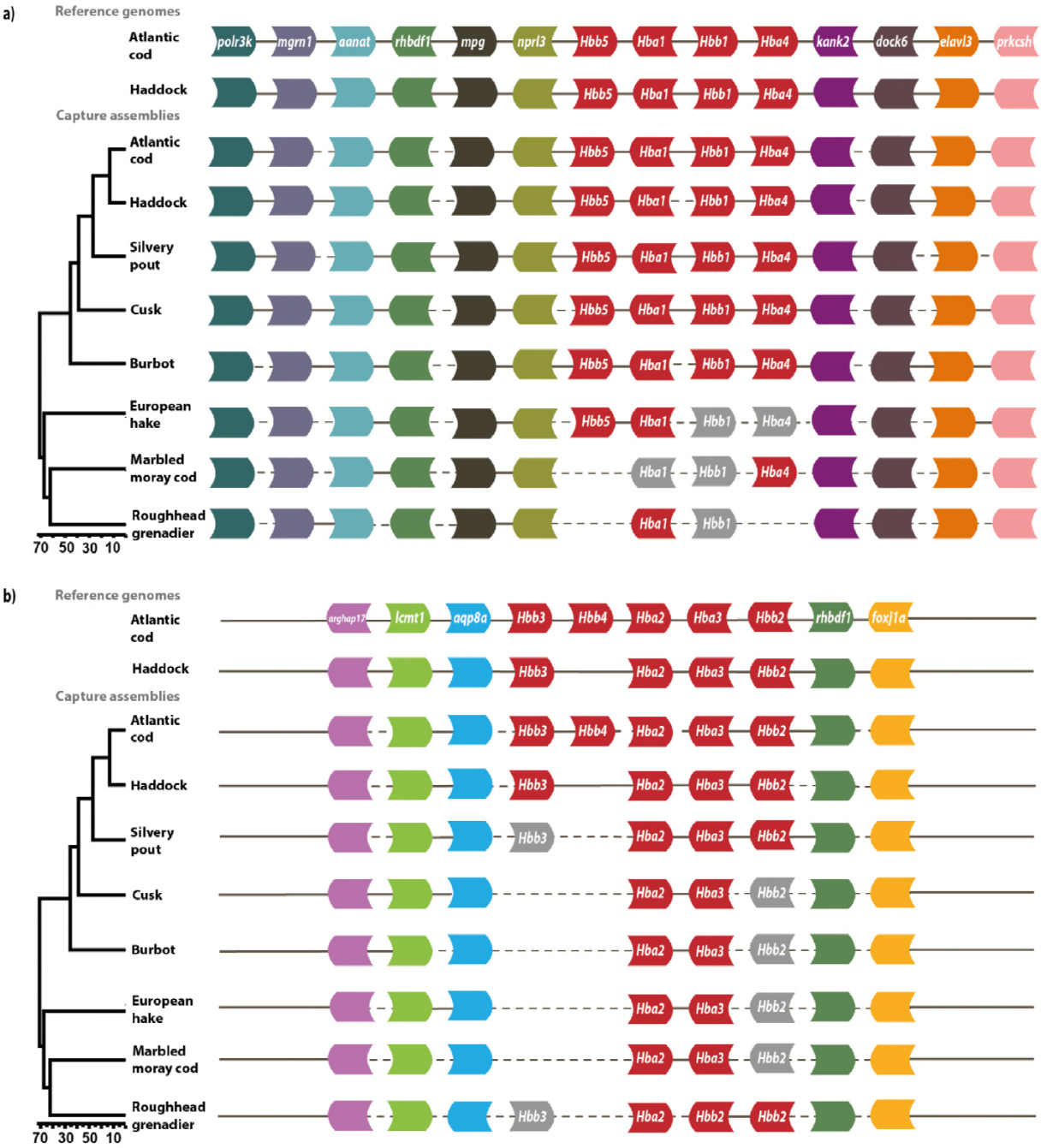
a)
Reference genomes
Atlantic cod
Haddock
Capture assemblies
Atlantic cod
Haddock
Silvery pout
Cusk
Burbot
European hake
Marbled moray cod
Roughhead grenadier
70 50 30 10
polr3k
mgrn1
aanat
rhbdf1
mpg
nprl3
Hbb5
Hba1
Hbb1
Hba4
kank2
dock6
elavl3
prkcsh
b)
Reference genomes
Atlantic cod
Haddock
Capture assemblies
Atlantic cod
Haddock
Silvery pout
Cusk
Burbot
European hake
Marbled moray cod
Roughhead grenadier
70 30 50 10
arghap17
lcmt1
aqp8a
Hbb3
Hbb4
Hba2
Hba3
Hbb2
rhbdf1
foxj1a

Figure 5

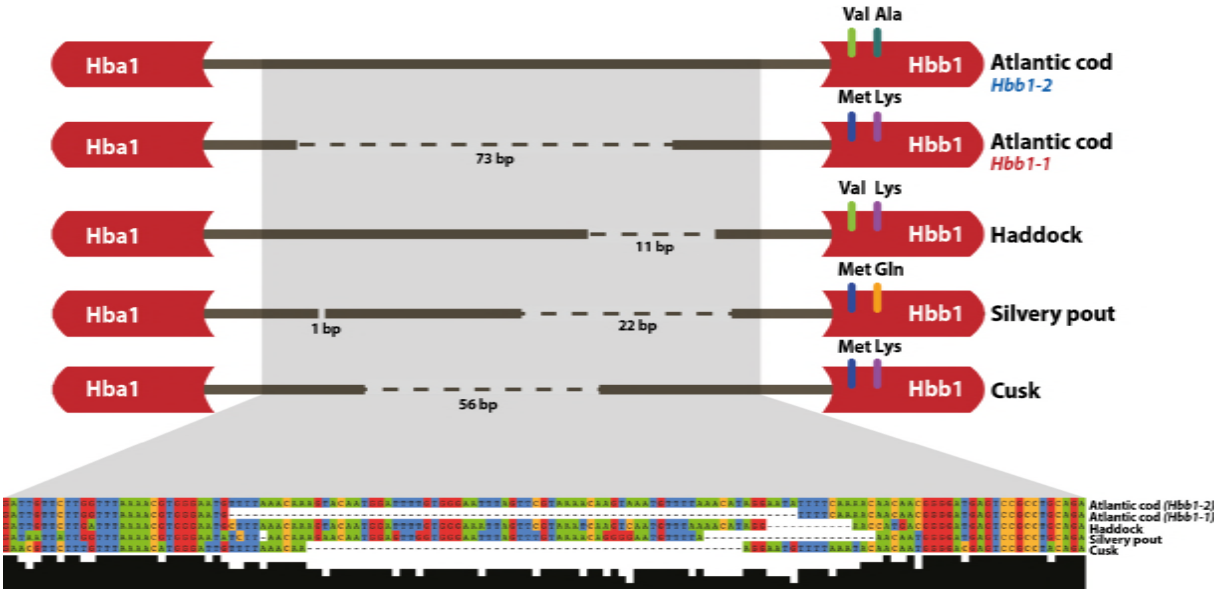
Hba1
Hbb1
Val Ala
Atlantic cod
Hbb1-2
Met Lys
Atlantic cod
Hbb1-1
73 bp
Val Lys
Haddock
11 bp
Met Gln
Silvery pout
1 bp
22 bp
Met Lys
Cusk
56 bp
Atlantic cod (Hbb1-2)
Atlantic cod (Hbb1-1)
Haddock
Silvery pout
Cusk

Figure 6

836 # Tables

837 **Table 1.** Number of reads and bases captured and sequenced for each species, and
838 number of utgs, largest utg and N50 in the assemblies.

Species	Latin name	Number of reads	Number of bases	Number of utgs	Largest utg (bp)	N50 (bp)
Atlantic cod	*Gadus morhua*	73005	217252583	278	79 020	7 728
Haddock	*Melanogrammus aeglefinus*	35573	107839552	227	52 433	7 227
Silvery pout	*Gadiculus argenteus*	69775	212519845	410	35 801	7 098
Cusk	*Brosme brosme*	55348	175883008	394	64 145	7 322
Burbot	*Lota lota*	56155	165360828	205	70 602	8 055
European hake	*Merluccius merluccius*	65661	180558336	311	31 558	6 523
Marbled moray cod	*Muraenolepus marmoratus*	52076	148100933	455	30 019	6 632
Roughhead grenadier	*Macrourus berglax*	46195	129085001	325	35 216	7122

839
840