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REVIEW PAPER

Genomic resources for flatfish research and their applications

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Running headline: FLATFISH GENOMICS

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Abstract

Flatfishes are a group of teleosts of high commercial and environmental interest but whose biology is still poorly understood. The recent rapid development of different 'omics' technologies is however enhancing the knowledge of the complex genetic control underlying different physiological processes of flatfishes. This review describes the different functional genomics approaches and resources currently available for flatfish research, and summarizes different areas where microarray-based gene expression analysis has been applied. The increase in genome sequencing data has also allowed the construction of genetic linkage maps in different flatfish species; these maps are invaluable for investigating genome organization and identifying genetic traits of commercial interest. Despite the significant progress in this field, the genomic resources currently available for flatfish are still scarce. Further intensive research should be carried out to develop larger genomic sequence databases, high-density microarrays, and more detailed, complete linkage maps, using second-generation sequencing platforms. These tools will be crucial for further expanding our knowledge of flatfish physiology, and we predict that they will have important impacts on wild fish population management, improved fish welfare, and increased productivity in aquaculture.

Key words: Pleuronectiformes; EST, microarray; genomics; gene expression; genetic linkage map

48 **INTRODUCTION**

49

50 Flatfish, members of the order Pleuronectiformes, are a relatively large group of
51 ray-finned fish with about 570 extant species (Nelson, 2006). The name of the order is
52 derived from the Greek πλευρά (pleura), meaning "rib" or "side", and νηκτόν (nekton),
53 meaning "swimming". These fish have both eyes in one side and lie on the opposite
54 side, they are benthic and carnivorous, and most are marine species, although some
55 species occur only in freshwater. Flatfish are an interesting group of teleosts because
56 they show a unique developmental process known as metamorphosis, during which one
57 eye migrates across the top of the skull to lie adjacent to the other eye on the opposite
58 side, while the body flattens and lies on the eyeless side (Okada *et al.*, 2001). This is
59 accompanied by drastic morphological and physiological changes and its molecular
60 regulation is still poorly understood (Power *et al.*, 2008). The reproductive processes of
61 flatfish are also of scientific interest since the males of some species show testis of a
62 semi-cystic type, an unusual type of spermatogenesis among teleosts in which
63 spermatocytes and spermatids are released into the seminiferous lumen where they
64 differentiate into spermatozoa (Yoneda *et al.*, 1998; García-López *et al.*, 2005). In
65 addition, many flatfishes show elaborate courtship behaviours that are necessary for
66 successful mating but the underlying mechanisms are largely unknown (Gibson, 2005).
67 A number of flatfishes, including the flounders (*Platichthys flesus* L.,
68 *Paralichthys olivaceus* Temminck & Schlegel, *P. lethostigma* Jordan & Gilbert,
69 *Pseudopleuronectes americanus* Walbaum), soles, (*Solea solea* L. and *S. senegalensis*
70 Kaup), turbot (*Scopthalmus maximus* L.), plaice (*Pleuronectes platessa* L.), and Atlantic
71 halibut (*Hippoglossus hippoglossus* L.), are also important food resources. Because of
72 their highly prized white flesh, the aquaculture of a few of these species has been

enhanced or developed during the last years (Imsland *et al.*, 2003; Conklin *et al.*, 2003; Naylor & Burke, 2005; Mori *et al.*, 2006). The aquaculture production of turbot is the highest among flatfishes, whereas that of Atlantic halibut is now successfully underway although improvements in efficiency remain a major goal (Naylor & Burke, 2005). For other species, such as the Senegalese sole or the flounders, the production is lower, and in the case of sole different aspects of their culture need to be optimized to allow a sustainable and profitable industrial production.

The management of wild, captive and reared flatfish populations is in general impaired by the limited knowledge of different aspects of their biology. Recently, research on flatfish physiology has increased significantly, and 'omics' technologies (genomics, proteomics, metabolomics) have been applied to better characterize reproduction, development, nutrition, immunity, and toxicology in these species. These technologies are powerful tools for investigating the genetic and molecular regulation of biological processes in a global manner, and therefore they are of great interest for flatfish research. The proteomic approaches that have been used to investigate diverse biological questions in model and non-model fish species, including flatfishes, have recently been reviewed (Fornè *et al.*, 2010). Therefore, the scope of this review is to highlight the genomics technologies that have been developed so far in flatfishes, and summarize the different research areas where they have been applied.

DEVELOPMENT OF GENOMICS TOOLS IN FLATFISH

GENOMIC LIBRARIES

Although the genomes of five well-known model fish species, zebrafish (*Danio rerio* Hamilton), medaka (*Oryzias latipes* Temminck & Schlegel), spotted green

pufferfish (*Tetraodon nigroviridis* Marion de Procé), Japanese pufferfish (*Takifugu rubripes* Temminck & Schlegel) and three-spined stickleback (*Gasterosteus aculeatus* L.), have been fully sequenced, those of commercially important species including flatfish, has lagged behind. With the advent of novel methods for high-throughput DNA sequencing, such as 454 pyrosequencing technology (see below), more than 1,000,000 reasonably long reads (300-500 nucleotides) can be achieved in a 10 h run. Large-scale sequence analysis of Atlantic cod, *Gadus morhua* L., has been performed (Johansen *et al.*, 2009) and this approach is currently being applied to the sequencing of selected BACs from Atlantic halibut (e.g., Mechaly *et al.*, 2010).

Significant work has recently been achieved with half-smooth tongue sole (*Cynoglossus semilaevis* Günther), a flatfish of great commercial importance in China. In order to better understand the sex-determining mechanism in this species, a W sex chromosome-specific library covering almost 1% of the chromosome was constructed by amplifying DNA isolated by laser capture microdissection (Wang *et al.*, 2009a). Sequence analysis of 518 clones revealed only 75 significant BLASTX and BLASTN hits, including 24 repetitive sequences. A fosmid library consisting of 49,920 clones (3.23 genome equivalents) has also been constructed for the female half-smooth tongue sole and end-sequencing of both 5' and 3' ends of 1,152 individual clones generated 2,247 sequences after trimming, with an average sequence length of 855 nucleotides. BLASTN searches of the nr and expressed sequence tag (EST) databases of GenBank, and BLASTX searches of the nr database, resulted in 259 (11.53%) and 287 (12.77%) significant hits ($E < e\text{-}5$), respectively. This fosmid library will be a useful resource for large-scale genome sequencing, physical mapping, and positional cloning (Wang *et al.*, 2009b) and aid in understanding sex-determination in this species.

EST SEQUENCING

In the last decade, the number of ESTs and species of flatfish represented in public databases (e.g., GenBank) has increased substantially (Table I). EST surveys of flatfish species have been performed using classical high-throughput sequencing techniques which are costly, and this may explain why for some species the number of sequenced ESTs remains quite low. Winter flounder (*Pseudopleuronectes americanus* Walbaum) and Japanese flounder (*P. olivaceus* Temminck & Schlegel) were two fish subjected to EST analysis starting over 10 years ago (Aoki *et al.*, 1999; Douglas *et al.*, 1999; Kono & Sakai, 2001; Arma *et al.*, 2005), but recent surveys include ESTs from the digestive system (Kim et al., unpub.), and immune tissues such as liver, spleen and head kidney of Japanese flounder stimulated by various pathogens (Hirono et al., unpub.). Approximately 1,000 5'-end sequenced ESTs have been obtained from each of eight different tissue-specific and five different developmental stage-specific normalized cDNA libraries of Atlantic halibut (Douglas *et al.*, 2007), and over 4,000 ESTs were obtained from 2-cell stage embryos, 1 day-old yolk sac larvae and fast skeletal muscle of juvenile fish of the same species (Bai *et al.*, 2007). More than 10,000 ESTs have been 3'-end sequenced from a multi-tissue normalized cDNA library of Senegalese sole from adult tissues, larval and juvenile stages, and undifferentiated gonads (Cerdà *et al.*, 2008b). Over 12,000 ESTs have also been generated from the liver, head kidney and spleen of turbot stimulated by nodavirus infection or polyIC (Park *et al.*, 2009), or by challenge with the bacterial pathogens *Aeromonas salmonicida* and *Philasterides dicentrarchi* (Pardo *et al.*, 2008). European flounder, *P. flesus* L., which is used in environmental monitoring has been extensively studied and ESTs have been sequenced from livers of fish exposed to a variety of toxic compounds such as heavy metals and other pollutants (Williams *et al.*, 2006).

148 ESTs have proved to be a valuable source of microsatellite markers for genetic
149 mapping. In Atlantic halibut, 129 microsatellites were identified by analysis of ESTs, 60
150 of which were polymorphic (Douglas *et al.*, 2007) and incorporated into a genetic
151 linkage map (Reid *et al.*, 2007). A total of 191 microsatellites were identified in the
152 turbot EST collection (Pardo *et al.*, 2008) of which 50 were present in contigs, thereby
153 allowing the identification of 11 putative polymorphic loci. In Japanese flounder, 5
154 microsatellites were identified in 260 ESTs generated from a muscle cDNA library, 3 of
155 which were also successfully amplified in turbot and half-smooth tongue sole (Liu *et*
156 *al.*, 2006). A similar approach was used to identified 25 microsatellites from 1,000
157 ESTs generated from a spleen cDNA library from half-smooth tongue sole (Liu *et al.*,
158 2007), 11 of which were useful in determining polymorphic loci. Several of these
159 microsatellites were also successfully amplified in turbot and flounder.
160 Single nucleotide polymorphisms (SNPs) can also be identified from EST surveys
161 if several individuals are used to make cDNA libraries, if there is sufficient redundancy
162 in the sequenced libraries, or if sequencing is sufficiently deep. Thus far in flatfish, this
163 has only been undertaken for turbot ESTs where 1,148 good quality SNPs were
164 identified among 9,256 ESTs (Pardo *et al.*, 2008).
165
166 MICROARRAYS
167 Gene expression profiling using microarrays has shed light on various
168 fundamental processes in fish, including immunity, gametogenesis and development,
169 nutrition, stress, and response/adaptation to environmental conditions (for reviews, see
170 Douglas, 2006; Cerdà *et al.*, 2008a; Goetz & MacKenzie, 2008; Miller & MacLean,
171 2008). In flatfish, single-species microarrays are available for five species (Table II). In
172 addition, multi-species microarrays have been developed for assessing fish stocks and

for response to environmental contaminants (Table III). In general, cross-species, or heterologous, microarrays have been used where microarrays for the species of interest are not available. Both oligonucleotide and cDNA probes have been used in flatfish microarray design and the probe density ranges from low (several hundreds) to very high (tens of thousands).

Two cDNA microarrays have been described for European flounder - the GENIPOL toxicogenomics microarray representing 3,336 unique EST clusters (Williams *et al.*, 2006), and an earlier version representing 3,352 unique sequences (Cohen *et al.*, 2007). The GENIPOL microarray has been useful in studying gene expression changes in European flounder in response to environmental toxicants or as a result of genetic adaptation (see below). This microarray was also used to assess cross-species hybridizations to transcriptomes of nine different fish, including flatfishes such as halibut, Japanese flounder and Senegalese sole (Cohen *et al.*, 2007; Osuna-Jimenez *et al.*, 2009). Using a bioinformatic approach, computed hybridisation efficiencies of 78.5-82.7% were obtained between European flounder, Japanese flounder and Atlantic halibut whereas efficiencies to the other teleosts tested were lower. Experimental validations showed hybridization efficiencies of 79% at sub-order taxonomic levels confirming that heterologous microarray analyses between closely related species can be performed.

Currently, oligonucleotide arrays are the preferred choice for flatfish microarray design given the lower cost and greater reproducibility of the expression data compared to that obtained from cDNA microarrays (Brennan *et al.*, 2004). Therefore, these platforms have recently been selected to design microarrays for different flatfishes (Cerdà *et al.*, 2008b; Douglas *et al.*, 2008; Kochzius *et al.*, 2008; Baker *et al.*, 2009; Millán *et al.*, 2009). For Senegalese sole, specific oligos were designed against the 3'

198 untranslated regions because of their general low conservation (Cerdà *et al.*, 2008b),
199 which permitted discrimination between paralogues arising from gene duplication
200 events in teleosts (Meyer & Peer, 2004). Thus, this microarray contains probes for 5,087
201 unigenes and allows the identification of specific isoforms within some gene families,
202 e.g., cyclins, vitellogenins, heat shock proteins, 40S and 60S ribosomal proteins.
203 Oligoarrays are also available for Atlantic halibut and turbot representing 9,277 and
204 2,716 unigenes, respectively (Douglas *et al.*, 2008; Millán *et al.*, 2009).
205 Multi-species microarrays, in which a single microarray is used to analyse the
206 different species under study, have been developed for comparative and ecological
207 genomics studies of fish (Kassahn, 2008). These platforms are, however, challenging
208 because limited signal intensity from fish with high sequence divergence, and variable
209 sequence divergence across different genes, must be accounted for in the experimental
210 design. Nevertheless, this approach may be useful when the expression of a few genes
211 across different species need to be studied, for which cross- hybridizing oligos can be
212 designed. This is the case, for instance for the multi-species microarray containing 65-
213 mer oligos designed to represent 24 genes involved in endocrine mechanisms from
214 many species, which has been used as a diagnostic tool to screen the effects of
215 environmental chemicals in the sentinel fish hornyhead turbot (*Pleuronichthys verticalis*
216 Jordan & Gilbert) (Baker *et al.*, 2009). Similarly, a multi-species microarray containing
217 23-27-mer oligos specific for mitochondrial 16S rDNAs of 11 species, including one
218 flatfish, *Scophthalmus rhombus* L., has been used for fish identification which can be
219 useful for correctly identifying fish eggs and larvae for stock assessment, and in food
220 control (Kochzius *et al.*, 2008). With more sequence information, greater refinement
221 and the planned production of a "Fish Chip" for approximately 50 species (see
222 Kochzius *et al.*, 2008), genotyping and population genetics studies in flatfish such as

those carried out on different chum salmon haplotypes (Moriya *et al.*, 2007) might be possible in the future.

Species-specific oligo microarray platforms are the most reliable for flatfish research. However, the current platforms available (e.g., sole, Atlantic halibut, turbot) contain still a relatively low number of probes (<10,000 unigenes) even though they were constructed from ESTs derived from different tissues. The limited number of represented genes makes these platforms challenging when the changes of the complete transcriptome of a tissue, particularly one from which ESTs were not derived, is to be investigated. Therefore, these platforms, although they may be suitable as a diagnostic tool for certain physiological conditions, are possibly not the best strategy for the discovery of genes and gene regulatory networks. Mass sequencing of the transcriptome through the use of next-generation sequencing platforms represents an alternative that may overcome some of these obstacles.

NEXT-GENERATION SEQUENCING PLATFORMS

Second-generation sequencing technologies allow massive-scale DNA sequencing at a low cost and are now driving biomedical and biological research (Mardis, 2008). These platforms include the 454 pyrosequencing system from Roche, which can yield 500 nucleotide reads, plus the Illumina/Solexa platform and the SOLiD platform from Applied Biosystems, which both yield shorter reads (~25-100 nucleotides). These platforms generate considerably more data per run and are more cost-effective in terms of price per nucleotide sequenced (Ansorge, 2009). The Illumina and SOLiD platforms are thus more suitable for model organisms or fish for which the whole genome has been sequenced, while the longer-read 454 platform is advantageous for *de novo*

247 sequencing in non-model fish such as flatfish, for which completely sequenced genomes
248 are not available.

249 The 454 technology can increase both genomic and EST sequence information,
250 and thus enlarge the number of unigenes represented in the microarrays. It also has the
251 advantage of combining gene discovery with expression profiling, and theoretically
252 provides information on the complete transcriptome rather than just the portion
253 represented on a microarray. Therefore, the labor-intensive construction of normalized
254 cDNA libraries required for microarray design and construction is not needed as high
255 throughput, in-depth sequencing provides an accurate estimate of gene expression
256 (Torres *et al.*, 2008). This technology has recently been used to sequence the
257 transcriptome of two commercially important fish species, the lake sturgeon (*Acipenser*
258 *fulvescens* Rafinesque) (Hale *et al.*, 2009) and Atlantic cod (Johansen *et al.*, 2009), and
259 it may be the preferred strategy for gene discovery in flatfish research.

260 The short-read Illumina/Solexa technology can be useful to investigate some gene
261 regulatory networks through the sequencing of microRNAs (miRNA) and other small
262 regulatory RNAs (Hafner *et al.*, 2008). miRNAs are small RNAs that bind to the 3'
263 UTR of mRNAs and control the accumulation of the target mRNAs in the cell, thereby
264 regulating a number of morphogenetic and developmental processes (Schier & Giraldez,
265 2006). The Illumina platform can potentially be used for miRNA discovery, surveying
266 and quantification in flatfish, although this approach has been used to date on only
267 model species with fully-sequenced genomes and still remains challenging (e.g., Chen
268 *et al.*, 2009; Rathjen *et al.*, 2009).

269

270 **GENE EXPRESSION PROFILING**

271

272 GAMETOGENESIS

273 Failure to complete ovarian maturation and ovulation is a common reproductive
274 dysfunction in cultured flatfishes even after hormone therapies (Mylonas & Zohar,
275 2001). Although in recent years a significant effort has been devoted to investigating the
276 endocrine basis of flatfish reproduction (e.g., Weltzien *et al.*, 2004; Kobayashi *et al.*
277 2008ab; Cerdà *et al.*, 2008c), the physiological mechanisms underlying reproductive
278 dysfunctions of cultured flatfish remain largely unknown. Transcriptome analyses have
279 been employed in some species, such the Senegalese sole, to obtain information on the
280 molecular basis of ovarian development (Tingaud-Sequeira *et al.*, 2009). In this study,
281 pairwise experiments using a sole-specific oligo array revealed the differential
282 expression of more than one hundred genes during ovarian growth, maturation and
283 ovarian follicle atresia. During ovarian growth (vitellogenesis), many up-regulated
284 ovarian transcripts had a putative mitochondrial function/location suggesting high
285 energy production, e.g., NADH dehydrogenase subunits, and increased antioxidant
286 protection, whereas other regulated transcripts were related to cytoskeleton and zona
287 pellucida organization, intracellular signalling pathways, cell-to-cell and cell-to-matrix
288 interactions, and the maternal RNA pool (Tingaud-Sequeira *et al.*, 2009). During
289 maturation, up-regulated transcripts in the ovary included ion transporters, e.g., Na^+-K^+-
290 ATPase subunits, which are probably required for oocyte hydration, as well as a vesicle
291 calcium sensor protein (extended synaptotagmin-2-A) that might be part of the
292 molecular pathways activated in the oocyte in preparation for fertilization.

293 During ovarian atresia, the process where ovarian follicles degenerate and are
294 resorbed (Saidapur 1978; Guraya 1986), two particularly interesting genes were found
295 to be highly up-regulated in Senegalese sole (Tingaud-Sequeira *et al.*, 2009). The first
296 of these was *apoc1,* encoding apolipoprotein C-I, which is part of chylomicrons and of

297 very low and high density lipoproteins involved in lipid transport in the bloodstream
298 (Jong *et al.*, 1999). The up-regulation of *apoc1* in follicular cells, as well as of
299 apolipoprotein A-I (*apoa1*) (Tingaud-Sequeira *et al.*, 2009), is thus consistent with a
300 role for these lipid transport molecules in mediating the ingestion and digestion of the
301 yolk by the follicular cells and further transport into the bloodstream (Saidapur, 1978;
302 Babin 1987). These data indicate the importance of lipid-metabolic processes during
303 follicular atresia in teleosts (Babin, 1987; Agulleiro *et al.*, 2007), and may provide
304 potential biomarkers for premature ovarian regression and abnormal embryo
305 development in cultured flatfish.

306 The other notable up-regulated gene in atretic ovarian follicles was *lect2* encoding
307 a leukocyte cell-derived chemotaxin 2 related protein (Tingaud-Sequeira *et al.*, 2009).
308 This transcript is related to mammalian *LECT2*, which encodes a protein with
309 chemotactic properties for human neutrophils (Yamagoe *et al.*, 1996). In atretic ovarian
310 follicles, blood cells such as erythrocytes and leukocytes (granulocytes) are often
311 observed invading the degenerating oocyte (Miranda *et al.*, 1999; Besseau & Faliex,
312 1994; Santos *et al.*, 2005). The up-regulation of *lect2*, which is most highly expressed in
313 theca and granulosa cells of atretic ovarian follicles, suggests an active mechanism for
314 the chemotaxin-mediated attraction of leukocytes to atretic follicles, where they act
315 synergistically with follicular cells in the resorption of the oocyte (Besseau & Faliex,
316 1994). Microarray analyses have therefore provided interesting insights into the
317 physiological activities that are important during this process.
318
319 LARVAL DEVELOPMENT AND NUTRITION
320 Microarrays have been used successfully in fish nutrigenomics studies as well as
321 for studying development (for review, see Douglas, 2006; Leaver *et al.*, 2008). For

322 flatfish, most studies have concentrated on Atlantic halibut. A microarray containing
323 50-mer oligonucleotides representing 9,277 unique Atlantic halibut genes has been used
324 to study early larval development (Douglas *et al.*, 2008). Principal component analysis
325 showed that the five different developmental stages analysed could be resolved from
326 one another. As larvae approached metamorphosis, genes involved in digestion
327 (trypsinogen, chymotrypsinogen), eye development and muscle structure (myosin,
328 tropomyosin, troponin) were up-regulated. Preliminary studies have also been reported
329 on the use of a small-scale cDNA microarray for studying gene expression changes in
330 whole larvae during flatfish metamorphosis (Power *et al.*, 2008). This study also found
331 myosin, trypsinogen and three genes involved in vision to be up-regulated during
332 metamorphosis.
333 Providing adequate nutrition to developing larvae and juveniles is a challenge in
334 the successful aquaculture of flatfish and microarrays have proved useful in assessing
335 gene expression changes in Atlantic halibut in response to dietary modifications. The
336 partial replacement of fish meal protein by soy or other plant proteins can result in
337 intestinal inflammation in some fish species, especially salmonids. In Atlantic halibut
338 juveniles, however, this condition was not observed when 30% soy protein was
339 included in the diet (Murray *et al.*, 2009a), although microarray analysis showed that
340 the expression of several immune markers and genes involved in detoxification was
341 increased. In contrast, genes involved in lipid transport were down-regulated, consistent
342 with previous reports of hypocholesterolemia in fish fed soy protein. Genes involved in
343 smooth muscle function were also down-regulated, indicating that intestinal muscle
344 metabolism and motility may have been affected. The replacement of live feeds by
345 artificial microdiets for the rearing of fish larvae, especially flatfish larvae, is a priority
346 for the aquaculture industry. Microarray analysis has been used to assess the effect of

347 introduction of microdiet to Atlantic halibut larvae 20 days post first feeding (Murray *et*
348 *al.*, 2009b). Although there was no significant difference in mortality in the microdiet-
349 fed group compared to the control group fed live feed over the 33 days of the study,
350 growth was limited and malpigmentation of the skin and eyes was more common.
351 Genes involved in metabolic processes were enriched, and their expression was
352 increased especially early after transfer to microdiet, as were genes involved in
353 detoxification and stress. Genes involved in replication, translation, cell proliferation
354 and cell structure were generally down-regulated, consistent with the lower growth of
355 the fish. As with most microarray studies, a significant number of unannotated genes are
356 differentially regulated in these studies, and identification of their functions may shed
357 light on crucial processes affected by dietary or developmental changes.
358
359 IMMUNOLOGY AND PATHOLOGY
360 Microarrays have been employed to better understand the immune response and
361 disease control in the commercially important flatfish Japanese flounder and turbot.
362 For the Japanese flounder, various cDNA microarrays containing subsets of
363 immune-relevant genes based on ESTs from a cDNA library derived from peripheral
364 blood leukocytes (Kurobe *et al.*, 2005) have been developed (Table II). A microarray
365 containing 871 unique elements was used to follow immune gene expression changes
366 over time (1, 3 and 6 h) in cultured head kidney cells stimulated by concanavalin A,
367 lipopolysaccharide (LPS), phorbol myristate acetate (PMA) and hirame rhabdovirus
368 infection (Kurobe *et al.*, 2005). Interestingly, different immune stimulants caused
369 different sets of genes to be regulated. As expected, LPS up-regulated a number of
370 inflammation-related genes; however, while PMA induced expression of transciption
371 factor AP-1 regulated cell proliferation genes, it mainly down-regulated genes, perhaps

372 through its inhibitory action on the transcriptional regulator, CEBPβ. Viral infection
373 resulted in a spike in expression of genes involved in early and cell-mediated immunity
374 at 3 h post-infection that mainly returned to normal after 6 h. This array was also used
375 to follow gene expression over time (1, 3 and 7 days) in head kidney cells of fish
376 injected with a plasmid expressing flounder IL-1β, a major inflammation-related
377 cytokine (Emmadi *et al.*, 2005). In this case, gene expression spiked at 1 day post-
378 injection and decreased over time. Approximately 10% of the 871 genes were
379 differentially regulated, with twice as many being up-regulated as down-regulated.
380 Genes for cytokines such as TNF and G-CSF as well as immunoglobulins, MHC Class I
381 antigens and members of the Toll and NF-kB signalling pathway were up-regulated.
382 Infections by viral hemorrhagic septicemia virus (VHSV) have devastated
383 salmonids and recently emerged among Japanese flounder. Hence, vaccine development
384 against this and other viral pathogens of fish is crucial. Two slightly different
385 microarrays have been used to evaluate vaccination of juveniles using recombinant
386 VHSV glycoprotein and a DNA vaccine encoding the glycoprotein (Byon *et al.*, 2005,
387 2006). The DNA vaccine conferred excellent protection, largely through the induction
388 of MX, whereas the recombinant glycoprotein vaccine was virtually ineffective, even
389 though it induced the expression of humoral defense-related genes and some non-
390 specific cellular defense-related genes. The DNA vaccine induced the most genes after
391 3 days; these included leukocyte-expressed genes involved in both the specific and non-
392 specific immune responses. Gene expression changes following vaccination of juveniles
393 against hirame rhabdovirus (HIRRV) using DNA vaccines for G and N proteins were
394 investigated using a microarray containing 796 unique elements (Yasuike *et al.*, 2007).
395 Interestingly, the two different vaccines induced different sets of genes which correlated
396 with protection; Type I interferon-induced genes were up-regulated by the vaccine

raised against the G protein and this vaccine was also protective whereas that raised against the N protein was not. This underscores the importance of the ability of vaccines to stimulate the type I interferon system.

The major bacterial pathogens affecting Japanese flounder are *Streptococcus iniae*, the Gram positive causative agent of streptococcosis, *Edwardsiella tarda*, the Gram negative causative agent of Edwardsiellosis, and *Mycobacterium* sp., the causative agent of mycobacteriosis. Vaccination against *S. inaie* and *E. tarda* using formalin-killed cells (FKC) was studied using a microarray containing 1,946 unique elements (Dumrongphol *et al.*, 2009) and an updated microarray containing 1,187 unique elements including additional novel genes from skin and liver ESTs (Matsuyama *et al.*, 2007a), respectively. The former study showed that *S. iniae* vaccination resulted in the dramatic up-regulation of a set of 8 genes at 3 h post-vaccination, whereas with *E. tarda* vaccination, a different set of 7 highly up-regulated genes were expressed at 1 day post-vaccination. In both cases, the transcript levels of most of the differentially regulated genes (six immune-related and 13 unknown) had returned to normal at 3 days post-vaccination. In the second study, fish were challenged after zero, one or two vaccinations with *E. tarda* FKC and sampled daily. As expected there were significant changes in gene expression in challenged fish that had been vaccinated as well as those that had not; however, in vaccinated fish there was a cluster of genes expressed throughout the sampling period that were uniquely up-regulated and may play a role in protection. Very recently, vaccination against mycobacteriosis using BCG and formalin-killed cells of *Mycobacterium* sp. was studied using a microarray containing 1,945 spots of unique genes including 215 immune-related genes (Kato *et al.*, 2010). BCG vaccination conferred protection against infection and also induced the expression of genes involved in both non-specific and adaptive immunity.

422 Parasite infection is also the cause of substantial losses to the Japanese flounder
423 industry. The monogenean parasite *Neoheterobothrium hirame* infects both wild and
424 cultured flounder, causing necrosis and inflammation at sites of attachment. Gene
425 expression changes in PBLs following infection by this parasite were monitored for
426 three weeks using a microarray containing 797 clones (Matsuyama *et al.*, 2007b).
427 Potential molecular biomarkers of infection were uncovered, including genes involved
428 in both non-specific (matrix metalloproteinases, CD20) and adaptive (MHC
429 components, immunoglobulins) immunity.
430 Similar disease problems have surfaced among turbot, necessitating a better
431 understanding of the immune system in this species and the development of vaccines.
432 The response of turbot to nodavirus infection and stimulation by the viral mimic,
433 polyIC, over a 72 h period was studied using a microarray containing 1,920 elements
434 representing 768 unique genes (Park *et al.*, 2009). MHCI genes and two interferon-
435 stimulated genes were up-regulated, consistent with the known role of these effectors in
436 viral immunity. Recently, a high-density microarray representing 2,716 genes from an
437 immune-related EST turbot database (Pardo *et al.*, 2008) was used to assess the
438 response of the spleen three days after infection by *A. salmonicida*, the causative agent
439 of furunculosis, and to identify candidate genes for resistance to pathogens (Millán *et*
440 *al.*, 2009). A set of 50 genes related to immunity and host defense were differentially
441 regulated, mostly positively, and with functions related to the innate immune response,
442 stress and/or defense response, transport and protein synthesis, processing or
443 degradation. Due to the layout of the microarray (eight microarrays fitted on each slide),
444 a hierarchical experimental design could be used to evaluate sources of technical and
445 biological noise in the differential gene expression of spleens from infected fish
446 compared to healthy fish.

447

448 TOXICOLOGICAL STUDIES

449 Flatfish are benthic teleosts that feed mostly on invertebrates and therefore are
450 impacted by sediment-associated toxicants, including endocrine disruptors, heavy
451 metals, polycyclic aromatic hydrocarbons, and dioxins. These species are thus good
452 candidates as sentinels for biological effects monitoring in inshore/estuarine waters
453 (Williams *et al.*, 2006; Baker *et al.*, 2009). Both multispecies and species-specific
454 microarrays (Tables II and III) have been successfully used in flatfishes, particularly in
455 the European flounder, to identify changes in gene expression after exposure to different
456 environmental pollutants (Sheader *et al.* 2006; Williams *et al.*, 2006, 2007, 2008; Diab
457 *et al.*, 2008; Nakayama *et al.*, 2008; Baker *et al.*, 2009). These studies have
458 demonstrated that key biological process disrupted by toxicants with different modes of
459 action can be identified by transcriptomics. The data may also permit discrimination
460 between classes of toxicants and the identification of molecular biomarkers for early
461 detection of pollutant responses in fish (Williams *et al.*, 2006, 2008), although
462 validation of these markers may require further biochemical, genetic and physiological
463 studies. Nevertheless, among the potential biomarkers that have been suggested in
464 flatfish are molecular chaperones, i.e., heat shock genes, oxidative stress responsive
465 elements, i.e., glutathione-S-transferases, peroxiredoxins, phase I and II metabolic
466 enzymes, i.e., cytochrome P450s, liver-derived egg proteins, i.e., vitellogenins and
467 choriogenins, and metallothionein, a metal ion sequestering protein (Williams *et al.*,
468 2006, 2008).

469 Most of the toxicogenomics studies carried out so far on flatfish used individual
470 toxicant treatment in laboratory-maintained fish and therefore the relevance of the
471 observed gene expression responses for natural populations is unclear. When

472 considering these populations, non-genetic effects may contribute to the observed
473 variation in gene expression making it very difficult to exclude influences from
474 maternal, early developmental or epigenetic effects resulting from interplay between
475 genetic background and parental/environmental variability. A study by Falciani *et al.*
476 (2008) partially addressed this issue and showed, by using a multivariate variable
477 selection coupled with statistical modelling methods, that gene expression signatures in
478 livers of flounders can predict their geographical site of origin, although the accuracy of
479 this system was limited to specific sites. This model used the expression profile of only
480 17 genes and was able to predict the site of origin of independent fish samples. The
481 future development of similar methods may prove very useful for evaluating the
482 susceptibility and adaptation to environmental pollutants in flatfishes.
483
484 POPULATION GENETICS
485 Neutral DNA-based markers, such as microsatellites, can be used to demonstrate
486 the existence of different subpopulations among marine fish populations at both macro
487 and microgeographical scales (e.g., Nielsen *et al.*, 2004; Jorgensen *et al.*, 2005). Such
488 genetic divergence may result in variations in gene expression, which is known to play
489 an important role in evolutionary processes of adaptive divergence among natural
490 populations (Nielsen *et al.*, 2009). The few available studies on natural fish populations
491 have suggested that variation in gene expression arises mainly from neutral genetic drift
492 (Oleksiak *et al.*, 2002; Whitehead & Crawford, 2006a). In relatively isolated
493 populations with low migration rates, such as those of the mummichog *Fundulus*
494 *heteroclitus* (L.), a strong correlation has been observed between genetic distance and
495 differences in gene expression (Oleksiak *et al.*, 2002; Whitehead & Crawford, 2006b).
496 However, in other marine species with higher migration rates and low level of genetic

497 differentiation, such as some flatfishes, actual variations in gene expression as an
498 adaptive response to a specific habitat are less well-known. This is however highly
499 relevant to the sustainable management and aquaculture of fish populations, including
500 flatfishes, since several studies have pointed out the high heritability of gene regulation
501 (Schadt *et al.*, 2003; Morley *et al*, 2004; Brem & Kruglyak, 2005; Whitehead &
502 Crawford, 2006a).

503 The determination of gene expression patterns through microarray-based
504 approaches provides more direct information on adaptive genetic divergence among
505 populations when compared with commonly used neutral genetic markers (Nielsen *et*
506 *al.*, 2009). This approach has been recently used to elucidate differences in gene
507 regulation between two flounder populations from the North Sea and Baltic Sea that,
508 apparently, are almost genetically identical based on microsatellite markers (Larsen *et*
509 *al.*, 2007). This study demonstrated that despite extremely low levels of neutral genetic
510 divergence, a high number of genes are significantly differentially expressed between
511 the two flounder populations maintained in a long-term reciprocal transplantation
512 experiment mimicking natural salinities. Several of the differentially regulated genes,
513 related to osmoregulation, heme biosynthesis and stress resistance, could be directly
514 linked to fitness traits (Larsen *et al.*, 2008). These findings suggest that flounders,
515 despite little apparent genetic divergence between populations, can adapt their gene
516 expression to local environmental conditions, and imply that such adaptation could be
517 common in other flatfishes with similar low levels of population subdivision (Larsen *et*
518 *al.*, 2007, 2008). However, the relationship of the local changes in gene expression with
519 single nucleotide polymorphisms (SNPs) has not been yet reported, and therefore a
520 more complete genetic characterization of flatfish populations is needed.
521

FLATFISH GENETIC MAPS

Genetic linkage maps are essential tools for investigating genome organization. They provide a brief outline of the genome of an organism based on the frequency of recombination between molecular markers and ideally generate the same number of linkage groups as the number of chromosomes. Typically, genetic maps are based on polymorphic markers such as microsatellites, variation at restriction sites, detected as restriction fragment length polymorphisms (RFLP) or amplified fragment length polymorphisms (AFLP), or SNPs. Microsatellites tend to be the most polymorphic of these markers while SNPs occur the most frequently in genomes. Construction of linkage maps requires hundreds of informative markers, ideally evenly spaced throughout the genome. While microsatellites are typically isolated by constructing libraries enriched for these sequences, EST libraries have been an excellent source of microsatellites for flatfish genetic maps (Liu *et al.*, 2006; Chen *et al.*, 2007; Liu *et al.*, 2007; Reid *et al.*, 2007; Bouza *et al.*, 2008; Kim *et al.*, 2009). Due to the well-established conservation of microsatellite loci among teleosts (Rico *et al.*, 1996), microsatellites are often informative in related species. This is also the case in the Pleuronectiformes (e.g. Liu *et al.*, 2006, 2007; Reid *et al.*, 2007) and thus all flatfish microsatellites are potentially useful for a given pleuronectid species. While SNPs have not yet been incorporated into any flatfish map, more than 1,000 were detected in turbot ESTs (Pardo *et al.*, 2008) and the application of next generation sequencing methods to flatfish genomics will undoubtedly allow the identification of massive numbers of useful genetic markers.

Currently, genetic linkage maps are available for four flatfish species: Japanese flounder (Coimbra *et al.*, 2003; Kang *et al.*, 2008), Atlantic halibut (Reid *et al.*, 2007),

turbot (Bouza *et al.*, 2007) and half-smooth tongue sole (Liao *et al.*, 2009). Maps for all species were constructed with microsatellites markers or a combination of microsatellite and AFLP markers (Table IV). The number of markers mapped range from 137 in sole to 604 in halibut. As would be expected, the number of markers mapped tends to correlate with the completeness of the map: maps with fewer markers tend to have more linkage groups than the haploid number of chromosomes and tend to have a number of small linkage groups with only a few (2-4) markers. The halibut map appears to be the most complete of these maps, although additional markers would clearly improve the coverage of all maps.

For two species (halibut and turbot), diploid gynogens have been used to map the position of the centromere for most of the linkage groups (Reid *et al.*, 2007; Martínez *et al.*, 2008). For halibut, all of the centromeres are located at or close to one end of the linkage group, with the centromere on linkage group AH-20, located at ~25 cM, being the most distant from the end of the linkage group. In turbot, the centromere locations for two linkage groups, LG-2 and LG-8, are metacentric and sub-metacentric, respectively, while the remaining mapped centromeres are acrocentric. For both species, these centromere locations are generally consistent with the karyotypes (Bouza *et al.*, 1994; Brown *et al.*, 1997), which provides support for the accuracy of these maps.

As in most fish species, flatfish also show differences in recombination rates between males and females, even though the overall map length is similar in the two sexes. Sex-associated recombination differences have been most carefully dissected in halibut, where it is evident that higher rates of recombination occur near the centromere in females (11-17 times the rate in males), but the difference decreases as one moves away from the centromere to the point where recombination is higher in males near the telomeres (2-3 times the rate in females) (Reid *et al.*, 2007). Sex-averaged maps (Bouza

572 *et al.*, 2007; Kang *et al.*, 2008; Liao *et al.*, 2009) tend to have inflated map lengths since
573 the high recombination regions of both sexes are incorporated into a single map.
574
575 CROSS-SPECIES GENOME COMPARISONS
576 The development of flatfish genetic maps provides the necessary resources for
577 genomic comparisons both within the flatfish and with other teleosts for which draft
578 genome sequences are available. The use of a large number of Japanese flounder
579 microsatellite markers in the Atlantic halibut genetic map allowed the identification of
580 linkage groups with common markers in the two species (Reid *et al.*, 2007). The
581 updated Japanese flounder map (Kang *et al.*, 2008) increases the correspondence
582 between the two species for some linkage groups due to the coalescence of pairs of
583 linkage groups in the original map or the removal of some markers from the map. Still,
584 both similarities and differences in marker order are seen in these two species.
585 For comparisons to draft genomes, marker sequences are typically compared to
586 the draft genome by BLAST to identify regions of similarity. Since not all markers give
587 clear BLAST results, these comparisons usually provide only a limited number of points
588 of comparison. Nevertheless, for both turbot and Atlantic halibut, multiple markers
589 from one linkage group map to a single chromosome of the spotted green pufferfish
590 whose genome has been sequenced (Bouza *et al.*, 2007; Reid *et al.*, 2007). Using the
591 pufferfish as an intermediate, correspondence between turbot and halibut linkage groups
592 can be inferred (Table V). This approach suggests, for example, that turbot LG-6
593 corresponds to halibut AH-3, since both contain markers mapping to pufferfish
594 chromosome 13. Clearly, a direct comparison between halibut and turbot is needed to
595 confirm this inference.

Conserved synteny blocks are indicative of orthologous genomic regions that arose from a common ancestral genome. Comparison of fish genomes with those of other vertebrates have revealed multiple synteny blocks conserved among all vertebrates as well as duplicated blocks that provide evidence for a fish-specific genome duplication (e.g., Elgar, 1996; Jaillon *et al.*, 2004; Naruse *et al.*, 2004). A recent investigation demonstrates a small, conserved synteny block between Atlantic halibut and five other teleosts in the region near the kisspeptin1 receptor gene (Mechaly *et al.*, 2010). In this region, gene order and organization is conserved in all six species for at least two protein-encoding genes as well as three microRNA genes. The region of synteny is extended in halibut, medaka and stickleback, where there are at least two more genes with the same organization, but not in zebrafish and the two pufferfish. Flatfish-specific synteny blocks will likely become evident as more flatfish genomic resources are developed.

APPLICATIONS OF GENETIC MAPS

Construction of a genetic linkage map provides additional useful information about the markers that have been mapped. Knowledge of the marker's linkage group and distance from the centromere can be useful in designing marker panels for population studies and aquaculture-related applications such as pedigree analysis or selected breeding programmes. The selection of a set of markers from different linkage groups will be more informative in these types of studies than a randomly selected group.

The main use of genetic maps is to provide a basis for the identification of quantitative trait loci (QTL). Most phenotypic characteristics of interest in aquaculture, such as growth rate or disease resistance, are complex and polygenic. A genetic map provides a basis for identifying regions of the genome that have a high correlation with

621 the desired trait and to use molecular markers from these regions for the selection of the
622 fish of interest. Methods and approaches for the identification of QTL and their use in
623 improving aquaculture broodstock have been described in a number of comprehensive
624 reviews (Canario *et al.*, 2008; Korol *et al.*, 2007, Liu & Cordes, 2004).

625 A QTL mapping approach was taken to identify markers in Japanese flounder
626 associated with resistance to lymphocystis disease (Fuji *et al.*, 2006). The authors
627 identified a marker on linkage group 15 that accounted for 50% of the phenotypic
628 variation in the group of fish that were screened. This marker was later used in a
629 marker-assisted breeding program to develop a lymphocystis disease-resistant line of
630 Japanese flounder (Fuji *et al.*, 2007).

631 A second example of QTL analysis in flatfish is the identification of a marker
632 associated with sex in turbot (Martínez *et al.*, 2009). In most fish, sex chromosomes are
633 not heteromorphic, with an obvious exception being half-smooth tongue sole (Chen *et*
634 *al.*, 2009), and thus molecular markers able to distinguish between sexes are of high
635 interest and have multiple applications in aquaculture. In turbot, sex is determined by a
636 ZW/ZZ system and thus a genome scan was used to identify markers linked to female
637 sex, and presumably the W sex-determining region. A marker near the centromere of
638 linkage group 5 was identified that was able to correctly sex 98% of the individuals in
639 four out of five families. Environmental or other minor genetic factors were thought to
640 account for the fish that could not be accurately sexed. This marker will clearly be
641 useful in assessing sex in turbot. We anticipate that additional QTL analyses for traits
642 important to flatfish aquaculture will be forthcoming.
643

644 ## CONCLUSIONS

645

646 The development of new genomic tools and approaches for flatfish, as well as for
647 other teleosts, is enhancing our knowledge of the biology and physiology of these fish,
648 which are of significant interest for commercial and environmental purposes. However,
649 the genomic resources currently available for flatfish are still scarce and should be
650 augmented by the development of more BAC libraries, larger EST databases, high-
651 density oligo microarrays, and more detailed, complete linkage maps. Second
652 generation sequencing methods will soon begin to replace these approaches as their read
653 lengths increase and new software is developed to handle the massive amounts of data
654 generated by them. These new sequencing technologies may also be a powerful tool for
655 the discovery of genes and gene regulatory networks, e.g., miRNAs, and will thus be
656 very useful for unravelling the genetic control of different flatfish biological processes.
657 This information will expand our basic knowledge of flatfish physiology and will
658 identify candidate genes as potential molecular biomarkers responsible for normal and
659 abnormal reproduction, larval development, stress, infections and pollutants. This will
660 lead to better management of wild populations, improved fish welfare, and increased
661 productivity in the aquaculture industry.
662
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666
667 **References**
668 Agulleiro, M. J., André, M., Morais, S., Cerdà, J. & Babin, P. J. (2007). High transcript
669 level of fatty acid-binding protein 11 but not of very low-density lipoprotein

receptor is correlated to ovarian follicle atresia in a teleost fish (*Solea senegalensis*). *Biology of Reproduction* **77,** 504-516.

Ansorge, W. J. (2009). Next-generation DNA sequencing techniques. *New Biotechnology* **25,** 195-203.

Aoki, T., Nam, B.-H., Hirono, I. & Yamamota, E. (1999). Sequences of 596 cDNA clones (565,977 bp) of Japanese flounder (*Paralichthys olivaceus*) leukocytes infected with Hirame rhabdovirus. *Marine Biotechnology* **1,** 477-488.

Arma, N. R., Hirono, I. & Aoki, T. (2005). Identification of genes expressed in the liver of Japanese flounder *Paralichthys olivaceus* by expressed sequence tags. *Fisheries Science* **71,** 504-518.

Babin, P. J. (1987). Apolipoproteins and the association of egg yolk proteins with plasma high density lipoproteins after ovulation and follicular atresia in the rainbow trout (*Salmo gairdneri*). *Journal of Biological Chemistry* **262,** 4290-4296.

Bai, J., Solberg, C., Fernandes, J. M. & Johnston, I. A. (2007). Profiling of maternal and developmental-stage specific mRNA transcripts in Atlantic halibut *Hippoglossus hippoglossus*. *Gene* **386,** 202-210.

Baker, M. E., Ruggeri, B., Sprague, L. J., Eckhardt-Ludka, C., Lapira, J., Wick, I., Soverchia, L., Ubaldi, M., Polzonetti-Magni, A. M., Vidal-Dorsch, D., Bay, S., Gully, J. R., Reyes, J. A., Kelley, K. M., Schlenk, D., Breen, E. C., Sásik, R. & Hardiman, G. (2009). Analysis of endocrine disruption in Southern California coastal fish using an aquatic multispecies microarray. *Environmental Health Perspectives* **117,** 223-30.

693 Besseau, L. & Faliex, E. (1994). Resorption of unemitted gametes in *Lithognathus*
694 *mormyrus* (Sparidae, Teleostei): a possible synergic action of somatic and
695 immune cells. *Cell & Tissue Research* **276,** 123-132.
696 Bouza, C., Sanchez, L. & Martinez, P. (1994). Karyotypic characterization of turbot
697 (*Scophthalmus maximus*) with conventional fluorochrome and restriction
698 endonuclease-banding techniques. *Marine Biology* **120,** 609-614.
699 Bouza, C., Hermida, M., Pardo, B. G., Fernandez, C., Fortes, G. G., Castro, J., Sanchez,
700 L., Presa, P., Perez, M., Sanjuan, A., de Carlos, A., Alvarez-Dios, J. A., Ezcurra,
701 S., Cal, R. M., Piferrer, F. & Martinez, P. (2007). A microsatellite genetic map of
702 the turbot (*Scophthalmus maximus*). *Genetics* **177,** 2457-2467.
703 Bouza, C., Hermida, M., Millan, A., Vilas, R., Vera, M., Fernandez, C., Calaza, M.,
704 Pardo, B. G. & Martinez, P. (2008). Characterization of EST-derived
705 microsatellites for gene mapping and evolutionary genomics in turbot. *Animal*
706 *Genetics* **39,** 666-670.
707 Brem, R. B. & Kruglyak, L. (2005). The landscape of genetic complexity across 5,700
708 gene expression traits in yeast. *Proceedings of the National Academy of Sciences*
709 *of the United States of America* **102,** 1572-1577.
710 Brennan, C., Zhang, Y., Leo, C., Feng, B., Cauwels, C., Aguirre, A. J., Kim, M.,
711 Protopopov, A. & Chin, L. (2004). High-resolution global profiling of genomic
712 alterations with long oligonucleotide microarray. *Cancer Research* **64,** 4744-
713 4748.
714 Brown, N. P., Bromage, N. R., Penman, D. J. & Shields, R. J. (1997). The karyotype of
715 the Atlantic halibut, *Hippoglossus hippoglossus* (Linnaeus). *Aquaculture*
716 *Research* **28,** 489-491.

717 Byon, J. Y., Ohira, T., Hirono, I. & Aoki, T. (2005). Use of a cDNA microarray to
718 study immunity against viral hemorrhagic septicemia (VHS) in Japanese flounder
719 (*Paralichthys olivaceus*) following DNA vaccination. *Fish & Shellfish*
720 *Immunology* **18,** 135-147.
721 Byon, J. Y., Ohira, T., Hirono, I. & Aoki, T. (2006). Comparative immune responses in
722 Japanese flounder, *Paralichthys olivaceus*, after vaccination with viral
723 hemorrhagic septicemia virus (VHSV) recombinant glycoprotein and DNA
724 vaccine using a microarray analysis. *Vaccine* **24,** 921-930.
725 Canario, A. V. M., Bargelloni, L., Volckaert, F., Houston, R. D., Massault, C. &
726 Guiguen, Y. (2008). Genomics toolbox for farmed fish. *Reviews in Fisheries*
727 *Science* **16**, 3-15.
728 Cerdà, J., Bobe, J., Babin, P. J. Admon, A. & Lubzens, E. (2008a). Functional genomics
729 and proteomic approaches for the study of gamete formation and viability in
730 farmed finfish. *Reviews in Fisheries Science* **16,** 54-70.
731 Cerdà, J., Mercadé, J., Lozano, J. J., Manchado, M., Tingaud-Sequeira, A., Astola, A.,
732 Infante, C., Halm, S., Viñas, J., Castellana, B., Asensio, E., Cañavate, P.,
733 Martínez-Rodríguez, G., Piferrer, F., Planas, J. V., Prat, F., Yúfera, M., Durany,
734 O., Subirada, F., Rosell, E. & Maes, T. (2008b). Genomic resources for a
735 commercial flatfish, the Senegalese sole (*Solea senegalensis*): EST sequencing,
736 oligo microarray design, and development of the Soleamold bioinformatic
737 platform. *BMC Genomics* **9,** 508.
738 Cerdà, J., Chauvigné, F., Agulleiro, M. J., Marin, E., Halm, S., Martínez-Rodríguez, G.
739 & Prat, F. (2008c). Molecular cloning of Senegalese sole (*Solea senegalensis*)
740 follicle-stimulating hormone and luteinizing hormone subunits and expression

741 pattern during spermatogenesis. *General and Comparative Endocrinology* **156,**
742 470-481.
743 Chen, S. L., Ma, H. Y., Jiang, Y., Liao, X. L. & Meng, L. (2007). Isolation and
744 characterization of polymorphic microsatellite loci from an EST library of turbot
745 (*Scophthalmus maximus*) and cross-species amplification. *Molecular Ecology*
746 *Notes* **7,** 848-850.
747 Chen, S. L., Tian, Y. S., Yang, J. F., Shao, C. W., Ji, X. S., Zhai, J. M., Liao, X. L.,
748 Zhuang, Z. M., Su, P. Z., Xu, J. Y., Sha, Z. X., Wu, P. F. & Wang, N. (2009).
749 Artificial gynogenesis and sex determination in half-smooth tongue sole
750 (*Cynoglossus semilaevis*). *Marine Biotechnology* **11,** 243-251.
751 Chen, X., Li, Q., Wang, J., Guo, X., Jiang, X., Ren, Z., Weng, C., Sun, G., Wang, X.,
752 Liu,Y., Ma, L., Chen, J.Y., Wang, J., Zen, K., Zhang, J. & Zhang, C. Y. (2009).
753 Identification and characterization of novel amphioxus microRNAs by Solexa
754 sequencing. *Genome Biology* **10**, R78.
755 Cohen, R., Chalifa-Caspi, V., Williams, T. D., Auslander, M., George, S. G., Chipman,
756 J. K. & Tom, M. (2007). Estimating the efficiency of fish cross-species cDNA
757 microarray hybridization. *Marine Biotechnology* **9,** 491-499.
758 Coimbra, M. R. M., Koretsugu, S., Kobayashi, K., Hasegawa, O., Ozaki, A., Naruse, K.
759 & Okamoto, N. (2003). A genetic linkage map of the Japanese flounder,
760 *Paralichthys olivaceus*, and observations on sex recombination. *Aquaculture* **204,**
761 203-218.
762 Conklin, D. E., Piedrahita, R. H., Merino, G. E., Muguet, J.-B., Bush, D. E., Gisbert, E.,
763 Rounds, J. & Cervantes-Trujano, M. (2003). Development of California halibut,
764 *Paralichthys californicus*, culture. *Journal of Applied Aquaculture* **14,** 143-154.

765 Diab, A. M., Williams, T. D., Sabine, V. S., Chipman, J. K. & George, S. G. (2008).
766 The GENIPOL European flounder Platichthys flesus L. toxicogenomics
767 microarray: application for investigation of the response to furunculosis
768 vaccination. *Journal of Fish Biology* **72,** 2154-2169.
769 Douglas, S. E., Gallant, J. W., Bullerwell, C. E., Wolff, C., Munholland, J. & Reith, M.
770 E. (1999). Winter flounder expressed sequence tags: Establishment of an EST
771 database and identification of novel fish genes. *Marine Biotechnology* **1,** 458-464.
772 Douglas, S. E. (2006). Microarray studies of gene expression in fish. *OMICS* **10,** 474-
773 489.
774 Douglas, S. E., Knickle, L. C., Kimball, J. & Reith, M. E. (2007). Comprehensive EST
775 analysis of Atlantic halibut (*Hippoglossus hippoglossus*), a commercially relevant
776 aquaculture species. *BMC Genomics* **8,** 144-155.
777 Douglas, S. E., Knickle, L. C., Williams, J., Flight, R. M. & Reith, M. E. (2008). A first
778 generation Atlantic halibut microarray: application to developmental studies.
779 *Journal of Fish Biology* **72,** 2391-2406.
780 Dumrongphol, Y., Hirota, T., Kondo, H., Aoki, T. & Hirono, I. (2009). Identification of
781 novel genes in Japanese flounder (*Paralichthys olivaceus*) head kidney up-
782 regulated after vaccination with Streptococcus iniae formalin-killed cells. *Fish &*
783 *Shellfish Immunology* **26,** 197-200.
784 Elgar, G. (1996) Quality not quantity: the pufferfish genome. *Human Molecular*
785 *Genetics* **5**, 1437-1442.
786 Emmadi, D., Iwahori, A., Hirono, I. & Aoki, T. (2005). cDNA microarray analysis of
787 interleukin-1b-induced Japanese flounder *Paralichthys olivaceus* kidney cells.
788 *Fisheries Science* **71,** 519-530.

789 Falciani, F., Diab, A. M., Sabine, V., Williams, T. D., Ortega, F., George, S. G. &
790 Chipman, J. K. (2008). Hepatic transcriptomic profiles of European flounder
791 (*Platichthys flesus*) from field sites and computational approaches to predict site
792 from stress gene responses following exposure to model toxicants. *Aquatic*
793 *Toxicology* **90,** 92-101.
794 Forné, I., Abián, J. & Cerdà, J. (2010). Fish proteome analysis: model organisms and
795 non-sequenced species. *Proteomics* **10**, 858-872.
796 Fuji, K., Kobayashi, K., Hasegawa, O., Coimbra, M. R. M., Sakamoto, T. & Okamoto,
797 N. (2006). Identification of a single major genetic locus controlling the resistance
798 to lymphocystis disease in Japanese flounder (*Paralichthys olivaceus*).
799 *Aquaculture* **254,** 203-210.
800 Fuji, K., Hasegawa, O., Honda, K., Kumasaka, K., Sakamoto, T. & Okamoto, N.
801 (2007). Marker-assisted breeding of a lymphocystis disease-resistant Japanese
802 flounder (*Paralichthys olivaceus*). *Aquaculture* **272,** 291-295.
803 García-López, A., Martínez-Rodríguez, G. & Sarasquete, C. (2005). Male reproductive
804 system in Senegalese sole *Solea senegalensis* (Kaup): anatomy, histology and
805 histochemistry. *Histology & Histopathology* **20,** 1179-1189.
806 Gibson, R. N. (2005). The behaviour of flatfishes. In: *Flatfishes: Biology and*
807 *Exploitation* (Gibson R. N., ed.), pp 213-239. UK: Blackwell Science.
808 Goetz, F. W. & MacKenzie, S. (2008). Functional genomics with microarrays in fish
809 biology and fisheries. *Fish Fisheries* **9,** 378-395.
810 Guraya, S. S. (1986). The cell and molecular biology of fish oogenesis. In:
811 *Monographs in Developmental Biology Vol. 18* (Sauer, H. W., ed.), pp 169-180.
812 Basel: Karger.

Hafner, M., Landgraf, P., Ludwig, J., Rice, A., Ojo, T., Lin, C., Holoch, D., Lim, C. & Tuschl, T. (2008). Identification of microRNAs and other small regulatory RNAs using cDNA library sequencing. *Methods* **44**, 3-12.

Hale, M. C., McCormick, C. R., Jackson, J. R. & Dewoody, J. A. (2009). Next-generation pyrosequencing of gonad transcriptomes in the polyploid lake sturgeon (*Acipenser fulvescens*): the relative merits of normalization and rarefaction in gene discovery. *BMC Genomics* **10**, 203.

Imsland, A. K., Foss, A., Conceição, L. E. C., Dinis, M. T., Delbare, D., Schram, E., Kamstra, A., Rema, P. & White, P. (2003). A review of the culture potential of *Solea solea* and *S. senegalensis*. *Reviews in Fish Biology and Fisheries* **13**, 379-407.

Jaillon, O., Aury, J.M., Brunet, F., Petit, J.L., Stange-Thomann, N., Mauceli, E., Bouneau, L., Fischer, C., Ozouf-Costaz, C., Bernot, A., Nicaud, S., Jaffe, D., Fisher, S., Lutfalla, G., Dossat, C., Segurens, B., Dasilva, C., Salanoubat, M., Levy, M., Boudet, N., Castellano, S., Anthouard, V., Jubin, C., Castelli, V., Katinka, M., Vacherie, B., Biémont, C., Skalli, Z., Cattolico, L., Poulain, J., De Berardinis, V., Cruaud, C., Duprat, S., Brottier, P., Coutanceau, J.P., Gouzy, J., Parra, G., Lardier, G., Chapple, C., McKernan, K.J., McEwan, P., Bosak, S., Kellis, M., Volff, J.N., Guigó, R., Zody, M.C., Mesirov, J., Lindblad-Toh, K., Birren, B., Nusbaum, C., Kahn, D., Robinson-Rechavi, M., Laudet, V., Schachter, V., Quétier, F., Saurin, W., Scarpelli, C., Wincker, P., Lander, E.S., Weissenbach, J. & Roest Crollius, H. (2004) Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature* **431**, 946-957.

Johansen, S. D., Coucheron, D. H., Andreassen, M., Karlsen, B. O., Furmanek, T., Jørgensen, T. E., Emblem, A., Breines, R., Nordeide, J. T., Moum, T., Nederbragt,

838 A. J., Stenseth, N. C. & Jakobsen, K. S. (2009). Large-scale sequence analyses of
839 Atlantic cod. *New Biotechnology* **25,** 263-271.
840 Jong, M. C., Hofker, M. H. & Havekes, L. M. (1999). Role of ApoCs in lipoprotein
841 metabolism: functional differences between ApoC1, ApoC2, and ApoC3.
842 *Arteriosclerosis, Thrombosis and Vascular Biology* **19,** 472-484.
843 Jorgensen, H. B. H., Hansen, M. M., Bekkevold, D., Ruzzante, D. E. & Loeschcke, V.
844 (2005). Marine landscapes and population genetic structure of herring (*Clupea*
845 *harengus* L.) in the Baltic Sea. *Molecular Ecology* **14,** 3219-3234.
846 Kang, J. H., Kim, W. J. & Lee, W. J. (2008). Genetic linkage map of olive flounder,
847 *Paralichthys olivaceus*. *International Journal of Biological Sciences* **4,** 143-149.
848 Kassahn, K. S. (2008). Microarrays for comparative and ecological genomics: beyond
849 single-species applications of array technologies. *Journal of Fish Biology* **72,**
850 2407-2434.
851 Kato, G., Kondo, H., Aoki, T. & Hirono, I. (2010). BCG vaccine confers adaptive
852 immunity against *Mycobacterium* sp. infection in fish. *Developmental*
853 *Comparative Immunology* in press doi:10.1016/j.dci.2009.08.013.
854 Kim, W. J., Kim, Y. O., Nam, B. H., Kong, H. J., Park, E. M., Kang, J. H., Lee, J. H. &
855 Kim, K. K. (2009). Development of 81 new polymorphic EST-derived
856 microsatellite markers for the olive flounder, *Paralichthys olivaceus*.
857 *Conservation Genetics* **10,** 1105-1111.
858 Kobayashi, T. & Andersen, Ø. (2008a). The gonadotropin receptors FSH-R and LH-R
859 of Atlantic halibut (*Hippoglossus hippoglossus*). 1: Isolation of multiple
860 transcripts encoding full-length and truncated variants of FSH-R. *General and*
861 *Comparative Endocrinology* **156,** 584-594.

862 Kobayashi, T., Pakarinen, P., Torgersen, J., Huhtaniemi, I. & Andersen, Ø. (2008b).
863 The gonadotropin receptors FSH-R and LH-R of Atlantic halibut (*Hippoglossus*
864 *hippoglossus*). 2. Differential follicle expression and asynchronous oogenesis.
865 *General and Comparative Endocrinology* **156,** 595-602.
866 Kochzius, M., Nölte, M., Weber, H., Silkenbeumer, N., Hjörleifsdottir, S.,
867 Hreggvidsson, G. O., Marteinsson, V., Kappel, K., Planes, S., Tinti, F., Magoulas,
868 A., Garcia Vazquez, E., Turan, C., Hervet, C., Campo Falgueras, D., Antoniou,
869 A., Landi, M. & Blohm, D. (2008). DNA microarrays for identifying fishes.
870 *Marine Biotechnology* **10,** 207-217.
871 Kono, T. & Sakai, M. (2001). The analysis of expressed genes in the kidney of
872 Japanese flounder, *Paralichthys olivaceus*, injected with the immunostimulant
873 peptidoglycan. *Fish & Shellfish Immunology* **11,** 357-366.
874 Korol, A., Shirak, A., Cnaani, **A.** & Hallerman, E.M. (2007). Detection and analysis of
875 quantitative trait loci (QTLs) for economic traits in aquatic species. In:
876 *Aquaculture Genome Technologies*. (Liu, Z., ed.), pp. 169-197. UK: Blackwell
877 Publishing.
878 Kurobe, T., Yasuike, M., Kimura, T., Hirono, I. & Aoki, T. (2005). Expression
879 profiling of immune-related genes from Japanese flounder *Paralichthys olivaceus*
880 kidney cells using cDNA microarrays. *Developmental and Comparative*
881 *Immunology* **29,** 515-523.
882 Larsen, P. F., Nielsen, E. E., Williams, T. D., Hemmer-Hansen, J., Chipman, J. K.,
883 Kruhøffer, M., Grønkjaer, P., George, S. G., Dyrskjøt, L. & Loeschcke, V. (2007).
884 Adaptive differences in gene expression in European flounder (*Platichthys flesus*).
885 *Molecular Ecology* **16,** 4674-4683.

886 Larsen, P. F., Nielsen, E. E., Williams, T. D. & Loeschcke, V. (2008). Intraspecific
887 variation in expression of candidate genes for osmoregulation, heme biosynthesis
888 and stress resistance suggests local adaptation in European flounder (*Platichthys*
889 *flesus*). *Heredity* **101,** 247-59.
890 Leaver, M. J., Bautista J. M., Björnsson B. T., Jönsson, E., Krey, G., Tocher, D. R. &
891 Torstensen, B. E. (2008). Towards fish lipid nutrigenomics: current state and
892 prospects for fin-fish aquaculture. *Reviews in Fisheries Sciences* **16**, 73-94.
893 Liao, X., Ma, H. Y., Xu, G. B., Shao, C. W., Tian, Y. S., Ji, X. S., Yang, J. F. & Chen,
894 S. L. (2009). Construction of a genetic linkage map and mapping of a female-
895 specific DNA marker in half-smooth tongue sole (*Cynoglossus semilaevis*).
896 *Marine Biotechnology* **11,** 699-709.
897 Liu, Z. J. & Cordes, J. F. (2004) DNA marker technologies and their applications in
898 aquaculture genetics. *Aquaculture* 238, 1-37.
899 Liu, Y. G., Zheng, M. G., Liu, L. X., Lin, H. & Wang, D. F. (2006). Five new
900 microsatellite loci for Olive flounder (*Paralichthys olivaceus*) from an expressed
901 sequence tag (EST) library and cross-species amplification. *Molecular Ecology*
902 *Notes* **6,** 371-373.
903 Liu, Y. G., Sun, X. Q., Gao, H. & Liu, L. X. (2007). Microsatellite markers from an
904 expressed sequence tag library of half-smooth tongue sole (*Cynoglossus*
905 *semilaevis*) and their application in other related fish species. *Molecular Ecology*
906 *Notes* **7,** 1242-1244.
907 Mardis, E.R. (2008). The impact of next-generation sequencing technology on genetics.
908 *Trends in Genetics* **23,** 133-141.
909 Martínez, P., Hermida, M., Pardo, B. G., Fernandez, C., Castro, J., Cal, R. M., Alvarez-
910 Dios, J. A., Gomez-Tato, A. & Bouza, C. (2008). Centromere-linkage in the

911 turbot (*Scophthalmus maximus*) through half-tetrad analysis in diploid
912 meiogynogenetics. *Aquaculture* **280,** 81-88.
913 Martínez, P., Bouza, C., Hermida, M., Fernandez, J., Toro, M. A., Vera, M., Pardo, B.
914 G., Millan, A., Fernandez, C., Vilas, R., Vinas, A. M., Sanchez, L., Felip, A.,
915 Piferrer, F., Ferreiro, I. & Cabaleiro, S. (2009). Identification of the major sex-
916 determining tegion of turbot (*Scophthalmus maximus*). *Genetics* (in press).
917 Matsuyama, T., Fujiwara, A., Nakayasu, C., Kamaishi, T., Oseko, N., Hirono, I. &
918 Aoki, T. (2007a). Gene expression of leucocytes in vaccinated Japanese flounder
919 (*Paralichthys olivaceus*) during the course of experimental infection with
920 Edwardsiella tarda. *Fish & Shellfish Immunology* **22,** 598-607.
921 Matsuyama, T., Fujiwara, A., Nakayasu, C., Kamaishi, T., Oseko, N., Tsutsumi, N.,
922 Hirono, I. & Aoki, T. (2007b). Microarray analyses of gene expression in
923 Japanese flounder *Paralichthys olivaceus* leucocytes during monogenean parasite
924 *Neoheterobothrium hirame* infection. *Disease of Aquatic Organisms* **75,** 79-83.
925 Mechaly, A. S., Vinas, J., Murphy, C., Reith, M. & Piferrer, F. (2010). Gene structure
926 of the kisspeptin-1 receptor-2 (Kiss1r-2) in the Atlantic halibut: insights into the
927 evolution and regulation of Kiss1r genes. *Molecular and Cellular Endocrinology*
928 **317,** 78-89.
929 Meyer, A. & van der Peer, Y. (2005). From 2R to 3R: evidence for a fish-specific
930 genome duplication (FSGD). *Bioessays* **27,** 937-945.
931 Millán, A., Gómez-Tato, A., Fernández, C., Pardo, B. G., Alvarez-Dios, J. A., Calaza,
932 M., Bouza, C., Vázquez, M., Cabaleiro, S. & Martínez, P. (2009). Design and
933 performance of a turbot (*Scophthalmus maximus*) oligo-microarray based on ESTs
934 from immune tissues. Mar. Biotechnol. (NY) in press. DOI 10.1007/s10126-009-
935 9231-0.

936 Miller, K. M. & MacLean, N. (2008). Teleost microarrays: development in a broad
937 phylogenetic range reflecting diverse applications. *Journal of Fish Biology* **72,**
938 2039-2050.
939 Miranda, C. L., Bazzoli, N., Rizzo, E. & Sato, Y. (1999). Ovarian follicular atresia in
940 two teleost species: a histological and ultrastructural study. *Tissue & Cell* **31,**
941 480-488.
942 Mori, T., Saito, S., Kishioka, C. & Arai, K. (2006). Aquaculture performance of triploid
943 barfin flounder *Verasper moseri*. *Fisheries Science* **72,** 270–277
944 Moriya, S., Sato, S., Azumaya, T., Suzuki, O., Urawa, S., Urano, A. & Abe, S. (2007).
945 Genetic stock identification of chum salmon in the Bering Sea and North Pacific
946 Ocean using mitochondrial DNA microarray. *Marine Biotechnology* **9,** 179-191.
947 Morley, M., Molony, C. M., Weber, T. M., Devlin, J. L., Ewens, K. G., Spielman, R. S.
948 & Cheung, V. G. (2004). Genetic analysis of genome-wide variation in human
949 gene expression. *Nature* **430,** 743–747.
950 Murray, H. M., Lall, S. P., Rajaselvam, R., Boutilier, L. A., Blanchard, B., Flight, R.
951 M., Colombo, S., Mohindra, V. & Douglas, S. E. (2009a). A nutrigenomic
952 analysis of intestinal response to partial soybean meal replacement in diets for
953 juvenile Atlantic halibut, *Hippoglossus hippoglossus*, L. *Aquaculture* (in press;
954 DOI 10.1016/j.aquaculture.2009.11.001).
955 Murray, H. M., Lall, S. P., Rajaselvam, R., Boutilier, L. A., Flight, R. M., Blanchard,
956 B., Colombo, S., Mohindra, V., Yufera, M. & Douglas, S. E. (2009b). Effect of
957 early introduction of microencapsulated diet to larval Atlantic halibut,
958 *Hippoglossus hippoglossus* L. assessed by microarray analysis. *Marine*
959 *Biotechnology* (in press; DOI 10.1007/s10126-009-9211-4).

960 Mylonas, C. C. & Zohar, Y. (2001). Use of GnRHa-delivery systems for the control of
961 reproduction in fish. *Reviews in Fish Biology and Fisheries* **10,** 463–491.
962 Nakayama, K., Kitamura, S., Murakami, Y., Song, J. Y., Jung, S. J., Oh, M. J., Iwata, H.
963 & Tanabe, S. (2008). Toxicogenomic analysis of immune system-related genes in
964 Japanese flounder (*Paralichthys olivaceus*) exposed to heavy oil. *Marine*
965 *Pollution Bulletin* **57,** 445-52.
966 Naruse, K., Tanaka, M., Mita, K., Shima, A., Postlethwait, J. & Mitani, H. (2004) A
967 medaka gene map: the trace of ancestral vertebrate proto-chromosomes revealed
968 by comparative gene mapping. *Genome Research* **14**, 820-828.
969 Naylor, R. & Burke, M. (2005). Aquaculture and ocean resources: raising tigers of the
970 sea. *Annual Review of Environment and Resources* **30,** 185-218.
971 Nelson, J. S. (2006). Fishes of the World 4th edition. New York: John Wiley & Sons.
972 Nielsen, E. E., Nielsen, P. H., Meldrup, D. & Hansen, M. M. (2004). Genetic population
973 structure of turbot (*Scophthalmus maximus* L.) supports the presence of multiple
974 hybrid zones for marine fishes in the transition zone between the Baltic Sea and
975 the North Sea. *Molecular Ecology* **13,** 585–595.
976 Nielsen, E. E., Hemmer-Hansen, J., Larsen, P. F. & Bekkevold, D. (2009). Population
977 genomics of marine fishes: identifying adaptive variation in space and time.
978 *Molecular Ecology* **18,** 3128-50.
979 Okada, N., Takagi, Y., Seikai, T., Tanaka, M. & Tagawa, M. (2001). Asymmetrical
980 development of bones and soft tissues during eye migration of metamorphosing
981 Japanese flounder, *Paralichthys olivaceus*. *Cell & Tissue Research* **304,** 59-66.
982 Oleksiak, M. F., Churchill, G. A. & Crawford, D. L. (2002) Variation in gene
983 expression within and among natural populations. *Nature Genetics* **32,** 261-266.

984 Osuna-Jimenez, I., Williams, T. D., Prieto-Alamo, M. J., Abril, N., Chipman, J. K. &
985 Pueyo, C. (2009). Immune- and stress-related transcriptomic responses of *Solea*
986 *senegalensis* stimulated with lipopolysaccharide and copper sulphate using
987 heterologous cDNA microarrays. *Fish & Shellfish Immunology* **26,** 699-706.
988 Pardo, B. G., Fernandez, C., Millan, A., Bouza, C., Vazquez-Lopez, A., Vera, M.,
989 Alvarez-Dios, J. A., Calaza, M., Gomez-Tato, A., Vazquez, M., Cabaleiro, S.,
990 Magarinos, B., Lemos, M. L., Leiro, J. M. & Martinez, P. (2008). Expressed
991 sequence tags (ESTs) from immune tissues of turbot (*Scophthalmus maximus*)
992 challenged with pathogens. *BMC Veterinary Research* **4,** 37.
993 Park, K. C., Osborne, J. A., Montes, A., Dios, S., Nerland, A. H., Novoa, B., Figueras,
994 A., Brown, L. L. & Johnson, S. C. (2009). Immunological responses of turbot
995 (*Psetta maxima*) to nodavirus infection or polyriboinosinic polyribocytidylic acid
996 (pIC) stimulation, using expressed sequence tags (ESTs) analysis and cDNA
997 microarrays. *Fish & Shellfish Immunology* **26,** 91-108.
998 Power, D. M., Einarsdóttir, I. E., Pittman, K., Sweeney, G. E., Hildahl, J., Campinho,
999 M. A., Silva, N., Sæle, Ø.; Galay-Burgos, M., Smáradóttir, H. & Björnsson, B. T.
1000 (2008). The molecular and endocrine basis of flatfish metamorphosis. *Reviews in*
1001 *Fisheries Science* **16,** 95-111.
1002 Rathjen, T., Pais, H., Sweetman, D., Moulton, V., Munsterberg, A. & Dalmay, T.
1003 (2009). High throughput sequencing of microRNAs in chicken somites. *FEBS*
1004 *Letters* **583**, 1422-1426.
1005 Reid, D. P., Smith, C. A., Rommens, M., Blanchard, B., Martin-Robichaud, D. &
1006 Reith, M. (2007). A genetic linkage map of Atlantic halibut (*Hippoglossus*
1007 *hippoglossus* L.). *Genetics* **177,** 1193-1205.

1008 Rico, C., Rico, I. & Hewitt, G. (1996) 470 million years of conservation of
1009 microsatellite loci among fish species. *Proceedings of the Royal Society London B*
1010 **263**, 549-557.
1011 Saidapur, S. K. (1978). Follicular atresia in the ovaries of nonmammalian vertebrates.
1012 *International Review of Cytology* **54,** 225-244.
1013 Santos, H. B., Rizzo, E., Bazzoli, N., Sato, Y. & Moro, L. (2005). Ovarian regression
1014 and apoptosis in the South American teleost *Leportinus taeniatus* Lutken
1015 (Caraciformes, Anostomidae) from the Sao Francisco Basin. *Journal of Fish*
1016 *Biology* **67,** 1446-1459.
1017 Schadt, E. E., Monks, S. A., Drake, T. A., Lusis, A. J., Che, N., Colinayo, V., Ruff, T.
1018 G., Milligan, S. B., Lamb, J. R., Cavet, G., Linsley, P. S., Mao, M., Stoughton, R.
1019 B. & Friend, S. H. (2003) Genetics of gene expression surveyed in maize, mouse
1020 and man. *Nature* **422,** 297-302.
1021 Schier, A. F. & Giraldez, A. J. (2006) MicroRNA function and mechanism: insights
1022 from zebrafish. *Cold Spring Harbor Symposium on Quantitative Biology* **71**, 195-
1023 203.
1024 Sheader, D. L., Williams, T. D., Lyons, B. P. & Chipman, J. K. (2006). Oxidative stress
1025 response of European flounder (*Platichthys flesus*) to cadmium determined by a
1026 custom cDNA microarray. *Marine and Environmental Research* **62,** 33-44.
1027 Tingaud-Sequeira, A., Chauvigné, F., Lozano, J., Agulleiro, M. J., Asensio, E. & Cerdà,
1028 J. (2009). New insights into molecular pathways associated with flatfish ovarian
1029 development and atresia revealed by transcriptional analysis. *BMC Genomics* **10,**
1030 434.
1031 Torres, T. T., Metta, M., Ottenwälder, B. & Schlötterer, C. (2008). Gene expression
1032 profiling by massively parallel sequencing. *Genome Research* **18,** 172-177.

1033 Wang, X., Zhang, Q., Ren, J., Jiang, Z., Wang, C., Zhueng, W. & Zhai, T. (2009a). The
1034 preparation of sex-chromosome-specific painting probes and construction of sex
1035 chromosome DNA library in half-smooth tongue sole (*Cynoglossus semilaevis*).
1036 *Aquaculture* **297,** 78-84.
1037 Wang, X., Zhang, Q., Sun, X., Chen, Y., Zhai, T., Zhuang, W., Qi, J. & Wang, Z.
1038 (2009b). Fosmid library construction and initial analysis of end sequences in
1039 female half-smooth tongue sole (*Cynoglossus semilaevis*). *Marine Biotechnology*
1040 **11,** 236-242.
1041 Weltzien, F.A., Andersson, E., Andersen, O., Shalchian-Tabrizi, K. & Norberg, B.
1042 (2004). The brain-pituitary-gonad axis in male teleosts, with special emphasis on
1043 flatfish (Pleuronectiformes). *Comparative Biochemistry and Physiology A*
1044 *Molecular and Integrative Physiology* **137,** 447-477.
1045 Whitehead, A., Crawford, D. L. (2006a). Variation within and among species in gene
1046 expression: raw material for evolution. *Molecular Ecology* **15,** 1197–1211.
1047 Whitehead, A. & Crawford, D. L. (2006b). Neutral and adaptive variation in gene
1048 expression. *Proceedings of the National Academy of Sciences of the United States*
1049 *of America* **103,** 5425–5430.
1050 Williams, T. D., Diab, A. M., George, S. G., Godfrey, R. E., Sabine, V., Conesa, A.,
1051 Minchin, S. D., Watts, P. C. & Chipman, J. K. (2006). Development of the
1052 GENIPOL European flounder (*Platichthys flesus*) microarray and determination
1053 of temporal transcriptional responses to cadmium at low dose. *Environmental*
1054 *Science and Technology* **40,** 6479-88.
1055 Williams, T. D., Diab, A. M., George, S. G., Sabine, V. & Chipman, J. K. (2007). Gene
1056 expression responses of European flounder (*Platichthys flesus*) to 17-beta
1057 estradiol. *Toxicology Letters* 168, 236-248.

1058 Williams, T. D., Diab, A., Ortega, F., Sabine, V. S., Godfrey, R. E., Falciani, F.,
1059 Chipman, J. K. & George, S. G. (2008). Transcriptomic responses of European
1060 flounder (*Platichthys flesus*) to model toxicants. *Aquatic Toxicology* **90,** 83-91.
1061 Yamagoe, S., Yamakawa, Y., Matsuo, Y., Minowada, J., Mizuno, S. & Suzuki, K.
1062 (1996). Purification and primary amino acid sequence of a novel neutrophil
1063 chemotactic factor LECT2. *Immunology Letters* **52,** 9-1.
1064 Yasuike, M., Kondo, H., Hirono, I. & Aoki, T. (2007). Difference in Japanese flounder,
1065 *Paralichthys olivaceus* gene expression profile following hirame rhabdovirus
1066 (HIRRV) G and N protein DNA vaccination. *Fish & Shellfish Immunology* **23,**
1067 531-541.
1068 Yoneda, M., Tokimura, M., Fujita, H., Takeshita, N., Takeshita, K., Matsuyama, M. &
1069 Matsuura, S. (1998). Reproductive cycle and sexual maturity of the anglerfish
1070 *Lophiomus setigerus* in the East China Sea with a note on specialized
1071 spermatogenesis. *Journal of Fish Biology* **53,** 164-178.

1072 TABLE I. Number of ESTs from flatfish in Genbank dbEST database as of Nov 2009 (Release 103009)

Species	Common name	Family	# ESTs
Hippoglossus hippoglossus L.	Atlantic halibut	Pleuronectidae	20836
Scopthalmus maximus L.	Turbot	Scophthalmidae	12427
Solea senegalensis Kaup	Senegalese sole	Soleidae	10631
Paralichthys olivaceus Temminck & Schlegel	Japanese (olive) flounder	Paralichthyidae	9983
Platichthys flesus L.	European flounder	Pleuronectidae	8396
Pseudopleuronectes americanus Walbaum	Winter flounder	Pleuronectidae	1483
Paralichthys lethostigma Jordan & Gilbert	Southern flounder	Paralichthyidae	596
Verasper variegatus Temminck & Schlegel	Spotted halibut	Pleuronectidae	524
Pleuronectes platessa L.	Plaice	Pleuronectidae	75

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TABLE II. Single-species microarrays designed to study differential gene expression in flatfish

Species	Number of Probes[a]/Unigenes	Probe type	Tissue source[b]	Target tissue[c]	Type of study	References
P. flesus L.[d]	13,824 (2X); 3,336 unigenes	cDNA	Liver	Liver	Cadmium chloride	Williams *et al.* (2006)
				Liver	17β-estradiol	Williams *et al.* (2007)
				Liver	Population genetics, adaptation	Larsen *et al.* (2007)
				Liver	Vaccination	Diab *et al.* (2008)
				Liver	Model toxicants	Williams *et al.* (2008)
				Liver	Ecotoxicogenomics	Falciani *et al.* (2008)
	11,060 (2X); 3,352 unigenes	cDNA	Liver	Liver	Species identification	Cohen *et al.* (2007)
	528 (4X)	cDNA	Liver	Liver	Cadmium chloride	Sheader *et al.* (2006)
H. hippoglossus L.	9,277 unigenes (4X)	Oligo (50 mer)	8 tissues, 5 developmental stages	Whole larvae	Larval development	Douglas *et al.* (2008)
				Whole larvae	Nutrigenomics	Murray *et al.* (2009b)
				Distal intestine	Nutrigenomics	Murray *et al.* (2009a)
S. senegalensis Kaup	5,087 unigenes (2X)	Oligo (60 mer)	6 tissues, 5 developmental stages	Ovary, whole larvae	Ovarian development, larval development	Cerdà *et al.* (2008b), Tingaud-Sequeira *et al.* (2009)
P. olivaceus Temminck & Schlegel	309 clones	cDNA	undisclosed	Kidney	Heavy oil	Nakayama *et al.* (2008)
	871 unigenes (2X)	cDNA	PBL[e]	Kidney cell line	Immunity	Kurobe *et al.* (2005)
				Kidney	Immunity	Emmadi *et al.* (2005)
	779 clones (2X)	cDNA	PBL	Kidney	Vaccination VHS	Byon *et al.* (2005)
	1,187 clones (2X)	cDNA	PBL	Kidney	Vaccination VHS	Byon *et al.* (2006)
	796 unigenes (2X)	cDNA	PBL	Kidney	Vaccination HIRRV	Yasuike *et al.* (2007)
	1,187 clones (2X)	cDNA	PBL, kidney, skin, spleen, liver	PBL	Vaccination *E. tarda*	Matsuyama *et al.* (2007a)
	1,946 unigenes	cDNA	PBL	Kidney	Vaccination *S. iniae*	Dumrongphol *et al.* (2009)
	797 clones (2X)	cDNA	PBL	PBL	Infection *N. hirame* parasite	Matsuyama *et al.* (2007b)
S. maximus L.	1,920 (3X); 768 unigenes	cDNA	Liver, gill, head kidney (challenged fish)	Kidney	PolyIC/nodavirus	Park *et al.* (2009)
	2,716 unigenes (5X)	Oligo (60 mer)	Liver, spleen, head kidney (challenged fish)	Spleen	Infection *A. salmonicida*	Millán *et al.* (2009)

1075 [a]Number of times each probe is spotted on the microarray.
1076 [b]Source of tissues used to generate EST data for design of microarray probes.
1077 [c]Source of tissue used for gene expression analysis.
1078 [d]Some additional clones from other flatfish included (see Williams *et al.* 2006 for details).
1079 [e]Peripheral blood leukocytes.

TABLE III. Heterologous and multi-species microarrays designed to study differential gene expression in multiple flatfish

Species	Number of Probes[a]/Unigenes	Probe type	Tissue source[b]	Target tissue[c]	Type of study	References
P. flesus L.	13,824 (2X); 3,336 unigenes	cDNA amplicon	*S. senegalensis*	Liver	Immunity/heavy metals	Osuna-Jimenez *et al.* (2009)
11 species (1 flatfish)	11 (4X) in 15 subarrays/slide	Oligo (23-27 mer)	Many	Gill, muscle mt 16S rDNA	Species identification	Kochzius *et al.* (2008)
Multiple species	24 endocrine genes in 6 subarrays/slide	Oligo (65mer)	*P. verticalis*	Liver	Endocrine disruptors in hornyhead turbot and zebrafish (*D. rerio* Hamilton)	Baker *et al.* (2009)

[a]Number of times each probe is spotted on the microarray.
[b]Source of tissues used to generate EST data for design of microarray probes.
[c]Source of tissue used for gene expression analysis.

TABLE IV. Summary of flatfish linkage maps

Species	# Chrom (1n)		# Markers		# Linkage groups		Map distance (cM)		References
			Microsat	AFLP	F	M	F	M	
P. olivaceus Temminck & Schlegel	24	Map 1	111	352	27	25	670	741	Coimbra *et al.* (2003)
		Map 2[a]	211	0	24		1001		Kang *et al.* (2008)
H. hippoglossus L.	24		258	346	24	24	1562	1460	Reid *et al.* (2007)
P. maxima L.	22	Consensus[a]	242	0	26		1343		Bouza *et al.* (2007)
		Haploid	172	0	27		1030		Bouza *et al.* (2007)
		Diploid	140	0	31	25	522	532	Bouza *et al.* (2007)
C. semilaevis Günther[a]	21		33	104	26		935		Liao *et al.* (2009)

[a]Sex-averaged maps

TABLE V. Correspondence of halibut and turbot linkage groups to spotted green pufferfish (*T. negroviridis* Marion de Procé) chromosomes[a]

Pufferfish chromosome	Turbot linkage group #	Halibut linkage group #
1	5, 7	6,21
2	2,3,13,21	**12**
3	13	5,9,20
4		13
5	1,25	**6**
6	17	24
7	13,18,UL	5,**22**
8	**2**	**10**
9	**10**	**2**
10	**3**,15	**7**
11		**15**
12	**12**,22	**14**
13	**6**	**3**
14	**23**	**1**
15	1	13,**18**
16	UL	12
17	19	17
18	**7**	**11**
19	**16**	**8**,17
20		
21		

[a]Bold type indicates linkage groups where more than one marker maps to the pufferfish chromosome.

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