Development of a Pacific oyster (*Crassostrea gigas*) 31,918-feature microarray: identification of reference genes and tissue-enriched expression patterns

Dheilly *et al.*
Development of a Pacific oyster (*Crassostrea gigas*) 31,918-feature microarray: identification of reference genes and tissue-enriched expression patterns

Nolwenn M Dheilly\(^1\)*, Christophe Lelong\(^1\), Arnaud Huvet\(^2\) and Pascal Favrel\(^1\)

**Abstract**

**Background:** Research using the Pacific oyster *Crassostrea gigas* as a model organism has experienced rapid growth in recent years due to the development of high-throughput molecular technologies. As many as 56,268 EST sequences have been sequenced to date, representing a genome-wide resource that can be used for transcriptomic investigations.

**Results:** In this paper, we developed a Pacific oyster microarray containing oligonucleotides representing 31,918 transcribed sequences selected from the publicly accessible GigasDatabase. This newly designed microarray was used to study the transcriptome of male and female gonads, mantle, gills, posterior adductor muscle, visceral ganglia, hemocytes, labial palps and digestive gland. Statistical analyses identified genes differentially expressed among tissues and clusters of tissue-enriched genes. These genes reflect major tissue-specific functions at the molecular level, such as tissue formation in the mantle, filtering in the gills and labial palps, and reproduction in the gonads. Hierarchical clustering predicted the involvement of unannotated genes in specific functional pathways such as the insulin/NPY pathway, an important pathway under study in our model species. Microarray data also accurately identified reference genes whose mRNA level appeared stable across all the analyzed tissues. *Adp-ribosylation factor 1* (*arf1*) appeared to be the most robust reference for normalizing gene expression data across different tissues and is therefore proposed as a relevant reference gene for further gene expression analysis in the Pacific oyster.

**Conclusions:** This study provides a new transcriptomic tool for studies of oyster biology, which will help in the annotation of its genome and which identifies candidate reference genes for gene expression analysis.

**Background**

The Pacific oyster *Crassostrea gigas*, is one of the world’s most economically important bivalves (4.2 million metric tons annual production, worth $3.5 billion) [1]. It is also one of the best characterized models for biochemical, molecular and genetics studies among the Lophotrochozoa. *Crassostrea gigas*, and other oyster species, play an important role in estuarine and marine coastal habitats, where increasing human activity is causing environmental degradation [2]. In these ecosystems, Pacific oysters suffer summer mortalities due to increased stresses and disease outbreaks [3]. At the other extreme, *C. gigas* can become invasive in new habitats (e.g. in northern Europe) [4]. The scientific rationale of obtaining a wide variety of sequences and developing genomic tools for *C. gigas* is to take advantage of its membership of the Lophotrochozoa, an understudied clade of bilateria. Its study will contribute to knowledge in the fields of functional, comparative and evolutionary genomics by throwing new light on genome function and diversity [5-9], the evolution of sexuality [10-13] or immunity [14-16]. In the context of environmental genomics, oysters might also prove to be an attractive model organism for understanding population responses to environmental stresses and adaptation through genetic change, as well as for deciphering the...
physiological bases of complex traits (growth, reproduction, survival) [17-20].

In July 2010, 56,268 EST sequences were compiled in the publicly accessible GigasDatabase version 6 housed at Sigenae (http://public-contigbrowser.sigenae.org:9090/Crassostrea_gigas/index.html) [21]. This genomic resource allows the development of genome wide microarrays for screening physiological traits and responses to the environment. DNA microarray technology is a high-throughput method for measuring the expression levels of thousands of genes simultaneously. This approach has been applied extensively to establish gene expression patterns in many organisms, including yeast, worm, human, fruit fly and rice [22-26]. Investigators have already analyzed gene expression in Crassostrea virginica and Crassostrea gigas using cDNA microarrays constructed from 6,780 [27] and 9,058 [21] genes. These microarrays were used to describe gene expression patterns in response to heat stress [28] and hypoxia [29], and to compare oysters that were resistant or susceptible to summer mortalities [21]. Since these studies were done, a large database of ESTs has become available, providing the information necessary to design a more comprehensive microarray suitable for characterizing genome-wide transcriptome profiles in Crassostrea gigas.

Here, for the first time, we describe the design of an oligo-microarray covering a total of 31,918 contig sequences, and its application to monitoring gene expression profiles in multiple tissues of adult oysters. In addition to the first obvious advantage offered by this new microarray, which is the significant increase in the number of contigs per array, this Agilent oligonucleotide microarray uses shorter probes (60 mer instead of an average mean of 413 bp in C. gigas cDNA arrays), resulting in higher density arrays and cheaper manufacture. In addition, with respect to cDNA arrays, oligonucleotide microarrays are reported to provide higher sensitivity and reproducibility [30]. This work aims to survey variation in gene expression across multiple oyster tissue types, to provide experimental evidence for gene function assignments, and to identify gene clusters related to tissue-specific processes. Knowledge of tissue-specific expression is a prerequisite to any studies of organism development, normal functioning and response to injury and disease. Furthermore, the comparison of significantly different transcriptomes allowed the identification of genes with stable expression across tissues. Quantification of gene expression has become a crucial step to investigating any molecular mechanism or physiological process in oysters. However, the quality of normalized quantitative RT-PCR data is dependent on the accuracy of the normalizer itself. To avoid misinterpretation of the differential expression profile of a target gene, it is therefore necessary to identify reliable reference genes and test their stable expression across the different experimental conditions tested. Because microarray screening simultaneously unravels the expression of a huge number of genes, it offers the opportunity to discover genes with transcriptional stability that can serve as pertinent internal reference standards for future quantitative RT-PCR studies on oysters.

The present genome-wide analysis of gene expression in C. gigas tissues does not only establish lists of genes expressed in the surveyed tissues, but also provides evidence for the level of expression of these genes in different tissues. Importantly, this approach may provide clues for elucidating the functions of genes underlying specific processes and identify candidate genes predicted to regulate traits of interest. This argument is particularly relevant for the unannotated ESTs, for which such a survey can provide clues to resolving the orphan status.

**Results and discussion**

**Robustness of C. gigas microarray data**

We designed a microarray using 31,918 oligonucleotide 60-mer probes representing the presently known genes in the Pacific oyster Crassostrea gigas (GigasDatabase, version 6). Of the 31,918 probes printed onto the array, 20,170 (63.2%) corresponded to annotated contigs in the GigasDatabase. This microarray is intended for use in studies of oyster transcriptomes at the whole-genome level. In our study, the microarray was used to analyze the transcriptome of 9 tissues using one-channel hybridization (Cy3). The custom microarray design and the raw and normalized data is available from the Gene Expression Omnibus (GSE26265; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26265).

The homogeneous hybridization of the arrays was first estimated by comparing the expression profiles of duplicated probes. Thirty-four probe pairs were printed onto the arrays (Additional file 1). Expression values were highly reproducible with a mean R² of 95.7% (Additional file 1). An R² of 98.4% was obtained by removing data points with expression values below background levels.

Next, to estimate the robustness of the microarray data, we compared the expression data obtained from different probes with identical annotation. Indeed, the oyster transcriptome is currently not completely sequenced and assembled and, therefore, some non full-length mRNAs are represented by numerous oligonucleotide probes printed on the array. This redundancy was used to confirm inter- and intra-array reproducibility. The consistency of the expression profiles obtained from different probes expected to come from the same mRNA confirmed the reproducibility of the data. For instance, among the 16 contigs originally annotated as similar to the myosin heavy chain of striated muscle from Aequipecten irradians (also known as Argopecten irradians)
the lowest measured correlation between microarray correlation increased to 93% when we removed the in the present work, reflects high microarray quality. ple probes corresponding to the same gene, as obtained of individually assessing microarray results and oligonu-ling expression profiles may thus be a more accurate way responding to the same contig and comparing their result-
data often disagree [33]. Using multiple probes corre-
xpression profiles showed very low gene expression values, close to or below background level, suggesting a low probe efficiency or a low expression in the different tissues. The most recent assembly of the C. gigas sequences (version 8, March 2011) constituted three contigs and one singleton from these 16 contigs (Figure 1B). Two contigs (BQ426757 and CU686207) encode two dif-
different myosin heavy chain isoforms (83% nucleotide identity). Contig CU686461 and singleton AM853364 encode two different isoforms of catchin transcript (86% nucleotide identity). Catchin is a protein present in mol-
suscan catch muscles, which is produced by alternative splicing of myosin heavy chain [31,32]. In our dataset, myosin heavy chain and catchin present very similar expression profiles across tissues and a higher expression in catch muscle (Figure 1A).

Such strong profile consistency was frequently observed in our dataset such as the 3 sequences that matched the uncharacterized protein ZK643.6 (Caenor-
habditis elegans) [GenBank: P30652]. Thus, our micro-
array data may prove useful in providing additional clues suggesting which contigs could constitute a single gene. The genes represented by multiple contigs can also be used to assess the reproducibility of the data and the homogeneity of the hybridization in future analysis. In Additional file 2 we provide a list of 200 putative genes significantly differentially expressed among tissues (ANOVA p < 0.05) and represented by 2 to 11 probes on the array. Over all contig pairs, we obtained a strong correlation of expression profiles (Mean $R^2 = 83.2\%$).

Quantitative RT-PCR was performed for four selected candidate reference genes and six tissue-enriched genes (primers listed in Additional file 3). The patterns of transcript abundance detected for these genes in the array and in quantitative RT-PCR showed extremely similar profiles (mean $R^2 \approx 85\%$) (Additional file 4). The correlation increased to 93% when we removed the hemocyte-enriched gene (BQ426482), which presented the lowest measured correlation between microarray and quantitative RT-PCR data (63%). Quantitative RT-PCR is commonly used as a validation tool for confirming gene expression results obtained from microarray analysis; however, microarray and quantitative RT-PCR data often disagree [33]. Using multiple probes corresponding to the same contig and comparing their resulting expression profiles may thus be a more accurate way of individually assessing microarray results and oligonu-
cleotide specificity. A strong correlation between multi-
ple probes corresponding to the same gene, as obtained in the present work, reflects high microarray quality.

Prevalent gene expression patterns
We applied principal component analysis (PCA) to the whole dataset. The PCA of all genes in the surveyed tis-
sues showed that datasets acquired from the same tissue type grouped together, indicating that there is a strong homogeneity in the gene expression pattern in each tissue type (Figure 2). Furthermore, tissues with similar functions had a lower scattering, as observed for the transcriptomes of gills and labial palps. Indeed, gills and labial palps are two pallial organs, both secreting mucus and possessing similar structures involved in particle selection, which is an essential part of bivalve filter-feeding.

It was not possible to extract a sufficient amount of total RNA for microarray hybridization from a single visceral ganglion or from hemolymph of a single individual. Therefore, we randomly pooled samples from 6 individuals. Pooling results in biological averaging for most genes. Consequently, the variation among samples in these two tissue categories is much lower than the variation among individuals for other tissues (Figure 2). However, Kend-
zierski et al. [34] demonstrated that the construction of a large number of pools enables the biological variation to be properly assessed and comparative studies to be made.

To further explore relationships among tissue samples, we performed a hierarchical clustering analysis on all the genes printed on the array. The results were extremely similar to those of the PCA analysis (Figure 3A). Gonads grouped together on a single branch, separated from all other tissues with 100% bootstrap support, demonstrating that the transcriptome of gonads (regardless of gender) is significantly different from the transcriptome of somatic tissues. Hierarchical clustering then further separated indi-
vidual gonad samples according to their gender. The data also showed that the digestive gland and adductor muscle clearly had different transcriptomes, with 100% bootstrap support. Mantle tissue clustered apart from visceral ganglion or from hemolymph samples with 60% bootstrap support. However, the hierarchical cluster analysis could not differenti-
te between labial palps and gills. Interestingly, labial palps and gills from the same individual always clustered together (1Pm with 1GIm, 5Pf with 5GIf and 6Pf with 6GIf) on the hierarchical clustering tree, once again demonstrating the strong similarities between these two tissues (Figure 3). Further analysis revealed that these organs co-expressed genes in relation with their common biological functions (see below).

Tissue-enriched gene expression
In the absence of a functional assay, tissue-specific gene expression features have often been viewed as indicators of tissue-specific function. In order to identify genes with differential tissue-enriched expression patterns, we initially performed a one-way ANOVA with a p-value
Figure 1  Expression profiles of contigs similar to P24733, the myosin heavy chain of striated muscle in GigasDatabase version 6 (A) and the new alignment of these contigs in GigasDatabase version 8 (B). A. Fourteen probes similar to P24733 (Myosin Heavy Chain from *Argopecten irradians*) show the same profiles of transcript abundance (line) and appear more abundant in mantle (M), Muscle (MS) and Hemocytes (HE) than in other tissues, and two probes did not show significant variation of signal intensity among tissue samples (dots). For each biological sample, numbers identify the individuals (individuals 1 to 8), letters identify the tissues (GI: Gills; G: gonad; DG: Digestive gland; M: Mantle; MS: Adductor muscle; P: Labial palps; GG: Visceral ganglia; HE: Hemocytes), followed by m for males (individuals 1, 7 and 8) and f for females (individuals 2, 3, 5 and 6); a, b, c and d correspond to pools of 6 individuals necessary to extract a sufficient amount of RNA for both visceral ganglia and hemocyte samples. B. The 16 contigs similar to P24733 have been re-assembled into 3 contigs [BQ426757; CU686461; CU686207] and one singleton [AM853364] in the most recent assembly of *C. gigas* transcriptome (GigasDatabase, version 8, February 2011).
A total of 7,586 contigs were differentially expressed between tissues (Table 1). These differentially expressed contigs were further filtered to determine whether they were overexpressed in a particular tissue. We required that the mean of the log_{10} normalized expression level of the replicates in a given tissue was at least 1.1 fold higher than the means in each of the other tissues (see Methods). A 1.1 fold increase of log_{10} values is equivalent to a 1.26 fold increase of the raw data. By this process, tissue-enriched contigs were identified for all tissues analyzed (Figure 3B).

The number of tissue-enriched contigs varied substantially, ranging from 23 contigs in labial palps to 471 in the digestive gland. The number of tissue-enriched contigs and the proportion of contigs annotated in the GigasDatabase are shown in Table 1. Figure 3B shows the heat map of the tissue-enriched contigs, and the complete list with their descriptions are given in Additional file 5. The highest percentage of contigs with annotation was obtained for genes specifically expressed in the digestive gland (51%). This is almost certainly because digestive processes are ancient and highly conserved. In digestive glands, many enzymes are widely distributed and conserved over a large range of species. For example, alpha-amylase, a key carbohydrate enzyme for starch digestion has been characterized from bacteria, fungi, plants and animals [35] and high sequence identity was observed for the domains responsible for its enzymatic activity [36]. It is also possible that there were qualitative differences in the original sequencing of mRNAs from the different tissues, which would result in poor BLAST matches. The constant updating of GigasDatabase will allow improvement of the sequence descriptions.

The annotated tissue-enriched contigs showed a strong relationship with the physiological functions of the corresponding tissues (Additional file 5). For example, numerous genes involved in digestion were highly expressed in the digestive gland (clh2, chia, plb1, pnlip, muc2, prss7, ...). 

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**Figure 2** 3D score plots using principal components (PC) identified by principal components analysis (PCA). Cumulative data of all 33 tissue samples for all oyster genes printed onto the array. Different individual samples from the same tissue type grouped together, and most tissue types were clearly different from each other.
Figure 3 Unsupervised hierarchical clustering of the gene expression profiles. (A) Classification of the 33 biological samples using unsupervised hierarchical clustering of the gene expression profiles (31,918 contigs) employing Pearson’s correlation. Numbers along the branches indicate bootstrap values after 100 iterations. Labial palp and gill tissue samples from the same individuals grouped together (1Pm and 1Bm; 5Pf and 5Bf; 6Pf and 6Bf). (B) Heat map of selected genes specifically overexpressed in different tissue types. Individual columns represent individual biological samples. The number of genes enriched in each tissue is indicated in Table 1. (C) Heat map of selected genes specifically over-expressed in two tissue types. (D) Expression of annotated and non-annotated genes potentially involved in regulating food-intake and energy balance. The cluster of genes was obtained using hierarchical clustering with Pearson’s correlation on the tissue-enriched genes. For each biological sample, numbers identify the individuals (individuals 1 to 8) and letters show the pools of 6 individuals (a, b, c and d), upper letters identify the tissues (G: Gonad; GI: Gills; P: Labial palps; M: Mantle; MS: Adductor muscle; DG: Digestive gland; GG: Visceral ganglia; HE: Hemocytes), followed by m for males (individuals 1, 7 and 8) and f for females (individuals 2, 3, 5 and 6).
The tissue distribution of contigs is shown in Table 1. The distribution of contigs is highly biased towards specific tissues, as evidenced by the number of genes annotated in the GigasDatabase. For example, the digestive gland contains the highest number of genes (471), followed by the adductor muscle (161), mantle (165), and labial palp (23). The female gonad contains the highest number of genes (268), followed by the male gonad (195), gills (61), and hemolymph (122).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Number of Genes</th>
<th>Number of Genes Annotated in GigasDatabase</th>
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</thead>
<tbody>
<tr>
<td>ANOVA significant</td>
<td>7,586</td>
<td>3,417 (45%)</td>
</tr>
<tr>
<td>Female Gonad</td>
<td>268</td>
<td>119 (44%)</td>
</tr>
<tr>
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<td>471</td>
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</tr>
<tr>
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<td>165</td>
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<tr>
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<td>161</td>
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<tr>
<td>Visceral ganglia</td>
<td>199</td>
<td>86 (43%)</td>
</tr>
<tr>
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<td>23</td>
<td>10 (44%)</td>
</tr>
<tr>
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<td>122</td>
<td>47 (39%)</td>
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Some functional clusters also showed similarities between tissues, such as male and female gonads, both of which expressed significantly higher levels of 341 genes than all other tissues, or labial palps and gills, which showed significantly higher expression of a common set of 101 genes. Hemocytes and visceral ganglia shared 54 overexpressed genes (Figure 3C). The complete list of dual tissue-enriched contigs and their descriptions are provided in additional file 6.

# Reference Genes

The term “housekeeping gene” is generally applied to genes ubiquitously expressed at the same level in all tissues. These constitute the basal transcriptome for the maintenance of basic cellular functions. Their uniform expression means they can be used as reference genes for quantitative RT-PCR [45]. Thus, we investigated the stability of gene expression levels to identify appropriate reference genes. To allow for comparisons of means and standard deviations, logarithmic normalized data were centered on their mean (fixed at 4). A candidate reference gene was defined as a gene that was not differentially expressed when tested by ANOVA (p-value < 0.05) and which had a coefficient of variation (CVarray, the ratio of the standard deviation to the mean) of <5%, a maximum fold change (MFC, the ratio of the maximum and minimum values observed within the dataset) of <1.1, and a mean expression level higher than the average.

References:

- Dheilly et al. BMC Genomics 2011, 12:468
- http://www.biomedcentral.com/1471-2164/12/468

**Table 1 Distribution of tissue-enriched contigs**

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expression on the array, i.e., > 4. One hundred and twenty-five unique genes were selected as reference candidate genes and ranked in order of increasing CVarray (Additional file 7). Among these, the 49 annotated reference gene candidates are shown in table 2.

Remarkably, none of the 3 most commonly used reference genes in oyster (actin, g3apdh and ef1α) ranked within the top 125 identified candidate reference genes. Actin and g3apdh had p-values < 0.05 when tested by ANOVA and were not ranked (Table 3). Ef1α did not show any significant variation when tested by ANOVA but, similarly to actin and g3apdh, it had an MFC > 1.1. Even if the MFC had not been taken into account, ef1α would still have ranked only 256th among the candidate genes (Table 3).

To demonstrate the importance of identifying appropriate reference genes, we designed primers for four of the 15 top-ranked novel reference genes (hkg4, hkg8, hkg12 and hkg14) and compared their mRNA levels estimated by quantitative RT-PCR with those of the three commonly used reference genes (actin, ef1α and g3apdh) in all tissue samples. To normalize data across samples, the mean of Ct values across all genes was calculated for each sample. The log values (mean Ct / Ct sample) were then compared for each gene. Box plots showing the minimum, maximum and mean value of Ct and log value (mean Ct/Ct sample) for each gene are shown in Figure 4A and 4B. In both datasets, hkg4, annotated as an adp-ribosylation factor 1 (arf1) in GigasDatabase, and g3apdh were the most stable genes, with coefficients of variation (CV; ratio of the standard deviation on the mean) of 0.04 for hkg4 and 0.05 for g3apdh. They also had the lowest CVs among all genes tested (Figure 4C). Applied to the raw data, Normfinder identified g3apdh as the best reference gene across the ensemble of the tissues, whereas it identified hkg4 as the best reference gene when considering normalized data. Using geNorm analysis on non-normalized expression levels, we determined the gene-stability expression measures (M) for the seven candidate reference genes. Again, hkg4 and g3apdh were the most stable reference genes, with an average expression stability value of 0.041 (Figure 4D). Arf1 has also been identified as a housekeeping gene in humans [46]. ADP-ribosylation factors are highly conserved 21 kDa GTPases involved in vesicular trafficking in all eukaryotes [47]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the most commonly used housekeeping genes for comparisons of gene expression data, though variability of GAPDH expression between human tissue types was recently shown [48]. In C. gigas, g3apdh was found to be significantly more expressed in the adductor muscle (ANOVA p < 0.05) and should, thus, be avoided when studying gene expression across different tissues. However, its expression appeared stable within tissues suggesting that it may be suitable for other experimental designs. We therefore identified ADP ribosylation factor 1 (arf1) as being a more relevant reference gene for use in normalization of real time PCR for tissue-expression studies than those typically used. In addition, the list of the 125 potential reference genes will clearly help in the choice of references genes in further gene expression studies, but we still emphasize the need to experimentally validate reference genes for specific experimental designs [45].

Conclusions

Research on gene function and oyster physiology using real time PCR and microarray is becoming increasingly important and widespread. Because of their economic importance, evolutionary position and use as a model organism in environmental genomics, more research is now being conducted on oyster species. A large EST project lead by French and US teams generated a genome-wide EST database, GigasDatabase, that is now freely available and housed on the Sigenae website. In the present study, the GigasDatabase was used to generate oligo-microarrays, providing the genomic tools necessary to undertake high-throughput studies of significantly important phenomena, such as summer mortalities in bivalves, host-pathogen interactions, reproduction and the effect of polyploidy on physiology of Pacific oysters. However, in order to make sense of gene expression data, we developed a 32 K microarray containing all known available contigs of C. gigas, thus aiming to represent the whole transcriptome. This study fulfilled 3 main goals. (1) It designed and presented a genome-wide oyster microarray that can now be employed in different gene expression studies in Pacific oysters (GSE 26265). The microarray is publicly available, following a request to the Agilent company and an authorization given by our team to share and use the oyster design. (2) It described the basic transcriptome of nine different tissues, providing important information for gene annotation and function prediction for genome sequencing. (3) It identified a large array of genes that can be used as reference genes to normalize gene expression data.

Methods

Animal sampling

Twenty-four adult oysters (3 years old) were sampled in July 2010 in Baie des Veys (Normandy, France). First, hemolymph was extracted from the adductor muscle using a 23-gauge needle attached to a 1-ml syringe, then gonad, gills, digestive gland, the posterior adductor muscle, labial palps, mantle and visceral ganglions were dissected. At this stage, pools of 6 individuals were randomly constituted for hemolymph and visceral ganglion samples. All samples were snap-frozen in liquid nitrogen, and stored at -80°C. Total RNA was extracted from individual tissues (3 female gonads, 3 male gonads, 4 gills, 4 digestive glands,
Table 2 The 49 annotated candidate reference genes ranked in order of their coefficient of variation (spot: location on microarray, ID genbank: accession number, stdev: standard deviation, description: best BLAST hit description as described in GigasDatabase, e value: best BLAST hit e-value, CV: coefficient of variation, max: maximum value, min: minimum value, MFC: maximum fold change, p value: one-way ANOVA P > 0.05)

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4 posterior adductor muscles, 4 labial palps, 4 mantles) or from pools of 6 individuals (4 pools of visceral ganglia and 3 pools of hemocytes) with Tri-Reagent kit (Sigma) and subsequently cleaned with Nucleospin RNA Clean-Up (Macherey Nagel) isolation columns. After the homogenisation of the N2 grinding powder in Tri-Reagent and the first centrifugation, the aqueous phase was extracted directly to isolate the total RNA on the column, as described in Table 2 The 49 annotated candidate reference genes ranked in order of their coefficient of variation (spot: location on microarray, ID genbank: accession number, stdev: standard deviation, description: best BLAST hit description as described in GigasDatabase, e value: best BLAST hit e-value, CV: coefficient of variation, max: maximum value, min: minimum value, MFC: maximum fold change, p value: one-way ANOVA P > 0.05) (Continued)

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CV indicates the coefficient of variation and equals the standard deviation divided by the mean. MFC indicates the maximum fold change, i.e., the ratio of the maximum and minimum values observed within the dataset.
Table 3 Ranking of 3 commonly used reference genes for quantitative real time PCR experiments on oysters (spot: location on microarray, ID genbank: accession number, stdev: standard deviation, description: best BLAST hit description as described in GigasDatabase, CV: coefficient of variation, max: maximum value, min: minimum value, MFC: maximum fold change, p value: ANOVA 1 factor, P > 0.05, NR: Not Ranked)

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CV indicates the coefficient of variation and equals the standard deviation divided by the mean. MFC indicates the maximum fold change, i.e., the ratio of the maximum and minimum values observed within the dataset.

by the manufacturer. Absence of trace genomic DNA was confirmed by gel electrophoresis. RNA concentrations were determined using a ND-1000 spectrophotometer (Nanodrop Technologies) at 260 nm, using the conversion factor 1 OD = 40 μg/ml RNA. RNA integrity was verified on an Agilent bioanalyzer using RNA 6000 Nano kits (Agilent Technologies), according to manufacturer’s instructions, without consideration for the RNA Integrity Number (RIN) [49]. Indeed, in molluscan RNA, the co-migration of the 28S rRNA fragments with the 18S rRNA prevented us from using the RIN, thus only the absence of RNA degradation can be considered [50-52]. A detailed description of the validation of RNA integrity in oysters is provided in Additional file 8. Samples were stored at -80°C until use.

Oligonucleotide microarray design and construction

The sequences used to design oligonucleotide probes were obtained from the publicly accessible GigasDatabase version 6, available on the Sigenae website (http://public-contigbrowser.sigenae.org:9090/Crassostrea_gigas/index.html) [21]. In July 2010, the final dataset contained 31,952 contigs assembled from 56,268 sequences selected from cDNA libraries derived from a wide variety of oyster tissues and developmental stages (Additional file 9). Contigs had previously been annotated with BLAST x and added to GigasDatabase [21]. From the resulting 31,952 contigs, 60-mer oligonucleotidic probes were designed using eArray software with the default parameters (Agilent Technologies). After repeat masking, low complexity filtering and similarity searches, 31,918 sequences contained exploitable probes. One probe per sequence was then printed onto a 4 x 44 K custom GE Microarray (Agilent Technologies). This automatic probe design generated 34 duplicated probes from highly similar sequences (Additional file 1). These probes were used to evaluate the reproducibility of hybridization (see results). Probes for negative controls were also printed onto the array in situ and used to test for low background before analysis (see Correction and Normalization). A new assembly (Gigas Database version 8) has been generated since the end of this study. The retrieval of sequences is possible using a Genbank name query, followed by .cg.8. All sequences used in this study are also available in Genbank and EMBL and are given in Additional file 9.

Oligonucleotide microarray hybridization

RNA Amplification, labeling and hybridization

For microarray hybridizations, 200 ng of total RNA was indirectly labeled with Cy3 using the Low Input Quick Amp labeling kit (Agilent Technologies), according to the manufacturer’s instructions. Qiagen RNeasy mini spin columns were used for purifying amplified RNA (aRNA) samples. After purification, aRNA amplification and dye incorporation rates were verified using a ND-1000 spectrophotometer (Nanodrop Technologies) and shown to lie between 200 and 500 ng/μL (aRNA concentration) and between 20 and 50 pmol/μg aRNA (dye incorporation).

Hybridization was performed using the Agilent Gene expression hybridization kit (5188-5242), as described by the manufacturer, with 1.65 μg of aRNA labeled with Cy3. Tissue samples were randomly hybridized onto 10 different slides, which were subsequently treated with Gene expression wash buffer solution (5188-5327; Agilent Technologies), and Stabilization and Drying solutions (5185-5979; Agilent Technologies). Finally, slides were scanned on an Agilent Technologies G2565AA Microarray Scanner system at 5 μm resolution, using default parameters.

Correction and Normalization

Feature extraction and data normalization were conducted with Agilent Feature Extraction software 6.1. (Agilent Technologies), using the default/recommended normalization method. Initial microarray testing revealed cross hybridization of C. gigas aRNA on the spiked-in probes. Thus Agilent spike-ins were not included in this study and are not recommended for future microarray analysis of C. gigas. The array contained 153 negative control spots with a mean gene expression of 0.84 (variance 19.6). Mean expression level of negative control probes was calculated for each tissue. The maximum mean obtained for each...
Figure 4 Expression stability of reference genes. Results of quantitative RT-PCR analysis are depicted as (A) Ct values measured or (B) Log (mean Ct/Ct sample) for each gene analyzed. Box plots graphically depict the minimum value, lower quartile, upper quartile and maximum value. (C) Coefficient of variation (CV; ratio of the standard deviation and the mean). (D) Average expression stability as calculated using geNorm. For details see Material and Methods. Genes are ordered according to increasing expression stability, identifying hkg4 and g3apdh as the best housekeeping genes for reference.
tissue × negative control probe was then used as a threshold level to determine *C. gigas* gene expression. According to this, 3,630 (11.4%) *C. gigas* probes had a gene expression below the background level in all tissue samples. A matrix of gene expression levels was generated, where each row corresponded to a different *C. gigas* oligonucleotide probe and each column to one tissue sample. The expression level of each contig was then logarithmically transformed, centered around 4 (the relative mean of all contigs within all tissues), and reduced so that relative variations rather than absolute values could be used for interpretation. After normalization, probes that showed gene expression at the background level had log₁₀ values ranging from 2.15 to 3.15, with a mean of 2.65.

**Microarray data analysis**

We initially applied a principal component analysis (PCA) using geneANOVA software [53] to assess the internal consistency of different transcriptional data sets and to obtain the proportion of variance for each principal component. Although component 1 had the highest proportion of variance (87.4%), it did not discriminate tissue samples from each other. All samples had exactly the same proportion of variance within this component.

PC1 would represent the great majority of genes that do not show tissue-related differential expression. Components 2, 3 and 4 discriminate the tissues and were used to draw a 3D scatter plot (XLstat; Addinsoft) and to organize the 33 tissue transcriptomes along the principal components. Hierarchical clustering using Pearson’s correlation was then performed using TMeV [54,55] and the results were compared to the PCA. The values of normalized signal intensities were further analyzed using one-way analysis of variance (ANOVA), with a p-value threshold of less than 0.01 and adjusted with Bonferroni correction (TMeV). Data were further filtered to determine whether their expression was specific to a tissue type. For each gene, the means of normalized expression from tissue replicates were calculated and compared to the means of normalized expression obtained for each of the other tissues.

**Quantitative RT-PCR**

For quantitative RT-PCR analysis, RNA samples were treated with DNase I (Promega; 1 U/µg total RNA) for 30 minutes at 37°C according to the manufacturer’s instructions. After DNase I treatment, absence of genomic DNA was confirmed by qPCR on a total RNA sample. Reverse transcription was then carried out using 1 µg of total RNA from each sample, 1 µg of random hexanucleotidic primers (Promega), 0.5 mM dNTPs and 200 U M-MLV reverse transcriptase (Promega) at 37°C for 1 hour in the appropriate buffer. Amplification reactions were performed in 1 x Absolute blue qPCR SYBRGreen fluorescentmix (Thermo scientific) with 5 ng of cDNA template and 300 nM of each primer in a final volume of 15 µL using an iCycler iQ™ thermocycler (Bio-Rad). The comparative threshold cycle (Cₜ) method was used to quantify copy number of the target gene in the tissues. Primer sequences used in this study are listed in additional file 3. The PCR amplification efficiency (E; E = 10(-1/slope)) for each primer pair was determined by linear regression analysis of a dilution series [56]. The specificity of the primer pairs was confirmed by melting curve analysis at the end of each qPCR run and each amplicon was verified by gel electrophoresis. For selected tissue-specific genes, the ΔCt was calculated using *arf1* (*adp-ribosylation factor 1*) as a reference gene and compared to the normalized expression values from the microarray analysis.

**Reference genes**

Genes expressed in all tissues with a mean normalized intensity that exceeded 4 (the mean of all normalized expression values) were considered as potential reference genes for quantitative RT-PCR. One-way ANOVA with a p-value threshold < 0.05 was then performed and candidate reference genes were defined as genes that were non-differentially expressed. For each gene, the coefficient of variation (CV, the ratio of the standard deviation to the mean) was calculated. CV was used to compare the degree of variation between the genes with different means of expression [57], and the Maximum Fold Change (MFC, ratio of the maximum and minimum values) was used to reflect the minor variations of those candidates within all biological samples [58]. Genes were thus ranked in order of increasing CV to identify the top most stable candidates.

Quantitative RT-PCR was performed to identify the best candidate control genes. The Ct values were determined at a specific threshold (fixed at 500 RLU). Ct values were normalized across samples by calculating the ratio of the Ct value to the mean of the Ct value obtained for all genes for a given sample and logarithmically transformed (base 10). The CV was calculated for each gene using normalized Ct values. Normfinder [59] was used on raw and normalized Ct values to measure stability values and identify the best housekeeping gene candidates. Finally, we used geNorm on raw Ct values to determine the expression stability (M value) [60]. Results obtained from the different methods were compared to validate the selection of ideal reference genes for comparative studies among tissues.

**Additional material**

Additional file 1: List of 34 duplicated contigs and the gene expression values obtained within each tissue. This table provides for each probe the sequence: Probe sequence, Row: Row on array, Col: Column on array, ProbeName, ContigName, Sigenae accession number.
GB_ACC: GenBank accession number. Description: Description from GigaS Database. GenBank accession number of corresponding duplicated probe, GI: Gills; G: gonad; DG: Digestive gland; M: Mantle; MS: Adductor muscle; P: Labial palp; GG: Visceral ganglia; HE: Hemocytes, R²: Coefficient of determination.

Additional file 2: List of 200 putative genes constituted of at least 2 different contigs that show extremely similar gene expression profiles. This table provides for each gene the ID, Genbank accession number, Description from GigaS Database, GeneName: gene name as cited in the text, mean: log normalized gene expression, log(mean + 1): log transformed mean expression, stdev: standard deviation of log normalized gene expression, CV: coefficient of variation, max: maximum value, Min: minimum value, MFC: maximum fold change, mean R²: mean Coefficient of determination.

Additional file 3: Housekeeping genes and tissue-enriched contigs selected for quantitative RT-PCR assay. This table provides for each contig the spot: location on microarray, ID, genbank accession number, length of primers (nucleotides), seq: 5’ to 3’ sequence of primer.

Additional file 4: Comparison of gene expression profiles of tissue-enriched contigs measured by microarray and quantitative RT-PCR analysis. Strong homologies were observed with a coefficient of correlation (R²) ranging from 65 to 93%. Letters identify the tissue (GI: Gills; G: gonad; DG: Digestive gland; M: Mantle; MS: Adductor muscle; P: Labial palp; GG: Visceral ganglia; HE: Hemocytes).

Additional file 5: Lists of tissue-enriched contigs: male gonad, female gonad, gills, labial palps, digestive gland, mantle, adductor muscle, visceral ganglion, hemocytes. This table provides for each the tissue: location on microarray, ID, genbank accession number, Description from GigaS Database, GeneName: gene name as cited in the text, mean: log normalized gene expression, stdev: standard deviation of log normalized gene expression, GI: Gills; G: gonad; DG: Digestive gland; M: Mantle; MS: Adductor muscle; P: Labial palp; GG: Visceral ganglia; HE: Hemocytes, SS: sum of squares, df: degrees of freedom, F: ratio of the model mean square to the error mean square, adj p value: adjusted Bonferroni’s correction p value.

Additional file 6: Lists of contigs gonad-enriched, gills- and labial palps-enriched, and gonad- and hemocyte-enriched. This table provides for each the tissue: location on microarray, ID, genbank accession number, Description from GigaS Database, GeneName: gene name as cited in the text, mean: log normalized gene expression, stdev: standard deviation of log normalized gene expression, GI: Gills; G: gonad; DG: Digestive gland; M: Mantle; MS: Adductor muscle; P: Labial palp; GG: Visceral ganglia; HE: Hemocytes, SS: sum of squares, df: degrees of freedom, F: ratio of the model mean square to the error mean square, adj p value: adjusted Bonferroni’s correction p value.

Additional file 7: 125 candidate reference genes identified within this study. This table provides for each the tissue: location on microarray, ID, genbank accession number, Description from GigaS Database, GeneName: gene name as cited in the text, mean: log normalized gene expression, stdev: standard deviation of log normalized gene expression, GI: Gills; G: gonad; DG: Digestive gland; M: Mantle; MS: Adductor muscle; P: Labial palp; GG: Visceral ganglia; HE: Hemocytes, SS: sum of squares, df: degrees of freedom, F: ratio of the model mean square to the error mean square, adj p value: adjusted Bonferroni’s correction p value.

Additional file 8: RNA integrity. Provides the method employed to measure RNA integrity in oyster samples.

Additional file 9: GigaSDatabase version 6. All sequences of GigaSDatabase version 6 in Fasta format.

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