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Molecular characterization and gene expression patterns of retinoid receptors, in normal and regenerating tissues of the sea cucumber, *Holothuria glaberrima*

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Abstract

Retinoic acid receptors (RAR) and retinoid X receptors (RXR) are ligand-mediated transcription factors that synchronize intricate signaling networks in metazoans. Dimer formation between these two nuclear receptors mediates the recruitment of co-regulatory complexes coordinating the progression of signaling cascades during developmental and regenerative events. In the present study we identified and characterized the receptors for retinoic acid in the sea cucumber *Holothuria* glaberrima; a model system capable of regenerative organogenesis during adulthood. Molecular characterizations revealed the presence of three isoforms of RAR and two of RXR as a consequence of alternative splicing events. Various analyses including: primary structure sequencing, phylogenetic analysis, protein domain prediction, and multiple sequence alignment further confirmed their identity. Semiquantitative reverse transcription PCR analysis of each receptor isoform herein identified showed that the retinoid receptors are expressed in all tissues sampled: the mesenteries, respiratory trees, muscles, gonads, and the digestive tract. During regenerative organogenesis two of the receptors (RAR-L and RXR-T) showed differential expression in the posterior segment while RAR-S is differentially expressed in the anterior segment of the intestine. This work presents the first description of the components relaying the signaling for retinoic acid within this model system.

Keywords

retinoic acid receptor; retinoid X receptor; retinoic acid; echinoderm

1. Introduction

The receptors for retinoic acid are members of the steroid and thyroid hormone superfamily of nuclear receptors (NR). These ligand-dependent transcription factors interact with DNA response elements controlling the enrollment of co-regulatory molecules that moderate

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Conflict of interest

The authors declare they have no competing interests.

transcriptional progression (Germain et al., 2006 a, b). The members of this superfamily activate various signaling cascades synchronizing developmental, metabolic, and physiological homeostasis in metazoans (Cotnoir-White et al., 2010). Signaling through the NR is mediated by steroid hormones (e.g. estrogen, glucocorticoids, vitamin D3), thyroid hormone (e.g. T3, T4), retinoids, or other small lipophilic molecules mainly derived from cholesterol (Sladek, 2011).

Retinoic acid signaling is relayed through two NR subfamilies: the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). RAR are members of the NR1B subfamily of NR (Kostrouchova and Kostrouch, 2014) and preferentially bind DNA as a RAR-RXR heterodimer (Mangelsdorf and Evans, 1995). Instead, RXR are members of the NR2B subfamily and can interact with DNA as self-sufficient homodimers or heterodimer alongside other NR such as vitamin D receptor, liver X receptor, or peroxisome proliferator-activated receptor (PPAR – Cotnoir-White et al., 2010), in addition to RAR. Apo (ligand-less) RAR-RXR heterodimers associate to retinoic acid response elements in an inhibitory manner that renders the promoter area inaccessible to the transcriptional machinery (Niederreither and Dollé, 2008; Al Tanoury et al., 2014). Binding of RA prompts the disassociation of the co-repressors stabilizing the nucleosome. This ultimately generates a binding area for the activation complex which stimulates chromatin remodeling allowing the recruitment of the transcriptional machinery into the targeted sequence (Chambon, 1996; Maden, 2007).

Orthologs for RXR have been identified all across the metazoan phylogeny (Escrivà et al., 2000), albeit the genes encoding for RAR appear to have been independently lost among some of its phyla (e.g. appendicularian tunicates, ecdysozoans, and cnidarians – Albalat, 2009; Gutierrez-Mazariegos et al., 2014). In addition to the chordates, RAR have been identified among ambulacrians (e.g. sea urchins, acorn worms) and lophotrochozoans (e.g. annelids, mollusks). Moreover, several isoforms for each RAR and RXR paralog have been identified across most metazoans as a result of differential promoter usage and alternative splicing (Zimmer et al., 1994). Predominantly up to three paralogs of both retinoid receptors can be recognized among vertebrates as a result of the whole genome duplication events characteristic of the lineage while non-vertebrates possess one of each (Dehal and Boore, 2005).

RA signaling regulates multiple signaling cascades synchronizing cellular processes such as axial patterning, cell cycle progression, or cell differentiation (Grenier et al., 2007; Tang and Gudas, 2011; Schubert et al. 2006). During early embryonic development retinoid signaling regulates cellular processes such as mammary gland and limb development, forebrain and hindbrain patterning, neural tube differentiation, and cardiogenesis (Cho et al., 2012; Rhinn and Dollé, 2012; Kam et al., 2012). Similarly, RA has been associated with regenerative processes. For example, in regenerating zebrafish fins, RA influences the size of the wound epidermis by stimulating apoptosis along the apical cap of the caudal fin (Ferretti et al., 1995; Geraudie et al., 1997). RA has also been proposed as the morphogen responsible for relaying positional patterning information across the proximodistal axis during amphibian limb regeneration (McEwan et al., 2011). Further studies based on DNA binding data revealed that RARβ and RXRα are attached to various sequences regulating cell cycle

progression (e.g. Cdk, cyclin – Liu et al., 2014). Moreover, chemical activation of RARβ or inhibition of cytochrome P450 family 26 (CYP26), a protein that irreversibly oxidize RA, gives rise to a limb duplication phenotype during *Xenopus*' limb regeneration (Cuervo and Chimal-Monroy, 2013).

Retinoic acid signaling was long thought to be a chordate innovation; as a result very little is known about its presence or function among invertebrates (Gutierrez-Mazariegos et al., 2014). As a first step in determining a possible role for this signaling pathway we have identified and confirmed the coding sequence (CDS) and untranslated regions (*UTR*) of both retinoid receptors in the sea cucumber *Holothuria glaberrima*, while assessing their expression along various adult tissues and different regenerative stages. The sea cucumber has proven to be a suitable model system to further our understanding of the cellular processes and molecular signals coordinating regenerative organogenesis of the gut and nervous tissue repair in adult organisms (San Miguel-Ruiz and García-Arrarás, 2007; Mashanov et al., 2008; Mashanov and García-Arrarás, 2011). Ultimately, this work paves the ground for a more detailed understanding on how retinoid signaling might be associated with the regenerative potential of this deuterostome invertebrate.

2. Materials and methods

2.1. Animal collection, maintenance, and autotomy induction

Adult sea cucumbers (*Holothuria glaberrima* species) 8-12 cm long, were collected from rocky intertidal zones of northeastern Puerto Rico (San Juan or Piñones). These were housed in aerated seawater aquaria at room temperature; 22°C (±2°C). All specimens were allowed to adapt to laboratory conditions overnight before initiating experimental procedures. Endogenous autotomy was initiated by injecting between 3-5 mL of 0.35 M KCl into the coelomic cavity (García-Arrarás et al., 1998). This caused the rupturing of the mesenteries holding the visceral mass and the subsequent expulsion of most the organs through the cloaca. Eviscerated individuals were allowed to regenerate for 3, 5, 7, and 14 days post evisceration (dpe) before dissecting and dividing the intestinal rudiment into the anterior, medial, and posterior regions.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from the selected tissues with a method previously described by Chomczynski (1993) for the simultaneous separation of RNA, DNA, and proteins using the TRI reagent (Sigma). The RNA phase is mixed with 70% ethanol and transferred to a column from the RNeasy Mini Kit (QIAGEN) for deoxyribonuclease (DNase) treatment (RNase-Free DNase Set – QIAGEN) and its subsequent isolation. The concentration and purity of the isolated RNA was then measure using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific). First strand DNA complementary to RNA (cDNA) was synthesized from 1 μg of total RNA with the Impromp-II Reverse Transcription System (Promega) and oligo $(dT)_{23}$ primer.

2.3. Sequence analyses: bioinformatics and phylogeny

Orthologs for both retinoid receptors, RAR and RXR, were probed against databases including over 5,173 expressed sequence tags (EST) from normal (uneviscerated) and regenerating (eviscerated) intestinal tissues (Rojas-Cartagena et al., 2007) as well as over 3 million RNAseq sequences obtained from nervous and intestinal tissues (Ortiz-Pineda et al., 2009; Mashanov et al., 2014). Significantly similar hits (e value > 10^{-30}) were subsequently queried against the non-redundant protein database within the NCBI webpage employing the Basic Local Alignment Search Tool (BLAST) to determine its identity/similarity and establish a proper reading frame. Primer pairs (Table 1) were designed from consensus segments to determine the full-length cDNA sequence thru multiple rounds of polymerase chain reaction (PCR) amplification and sequencing. PCR amplification was performed combining 0.15 mM of deoxyribonucleoside triphosphate (dNTP), 0.15 mM of each primer pair, 2.25 mM of $MgCl_2$, 0.1 U of Taq polymerase, 1.0 μL of the synthesized cDNA, and MilliQ water up to a final volume of 20.0 μL. PCR amplification involved an initial denaturation step at 94°C for 120 sec followed by 32-35 cycles of a 3-steps amplification condition that included a denaturation step of 45 sec (94°C), a primer-annealing step for 45 sec (56-60°C), and an extension step for another 45 sec (72°C). A final 10 min extension step (72°C) was included following the primer-annealing cycles. The samples were subsequently sequenced at the Sequencing & Genotyping Facility (UPR-RP) and the resulting information was later deposited into the GeneBank repository under accession numbers MF002011 (RAR-L), MF002010 (RAR-M), MF002012 (RAR-S), MF002013 (RXR-F) and MF002014 (RXR-T). Internet based prediction programs, SMART (Letunic et al., 2009) and InterProScan (Zdobnov and Apeweiler, 2001), were used to search for conserved domains of the predicted protein sequences. Geneious 9.1 (www.geneious.com) was subsequently used to develop the multiple sequence alignments and maximum likelihood phylogenetic trees (PhyML – Guindon and Gascuel, 2003). Information of the sequences used in the latter two analyses is included in the table Supplementary data S1. A web-based *UTR* analysis was performed with the UTRScan tool from the Institute of Biomedical Technologies.

2.4. Gene expression profile

Normal and regenerating individuals were sacrificed and dissected across the dorsoventral ambulacra to collect tissues. Adult tissues analyzed include: mesenteries, respiratory trees, muscles, gonads, and the esophagus, descending small intestine, the region connecting the ascending small intestine to the large intestine, and large intestine of the digestive tract. The anterior, medial and posterior regions of the regenerating intestine were dissected from animals at different stages post evisceration. Samples collected during the early regenerative stages were pooled since the intestinal tissue from one individual does not yield enough RNA concentration for the subsequent cDNA synthesis step. The intestinal primordia from three or two individuals were respectively pooled to obtain one sample of the 3 dpe and 5 dpe regenerative stages; meanwhile one intestinal primordial was used per sample for more advanced regenerative stages. Semiquantitative expression was assessed from the optical density values (Quantity One 4.6.6 – BioRad) of the images taken with the Molecular Imager ChemiDoc XRS+ (BioRad) from the samples bands in the electrophoresis gels. This data was normalized against the optical density values of NADH dehydrogenase subunit 5

since previous work has revealed its expression is not altered in normal and regenerating intestinal tissue (Rojas-Cartagena et al., 2007; Ramírez-Gómez et al., 2009). The PCR products employed during the gene expression profile were collected at a cycle depicting the beginning of their logarithmic phase. Normal individuals were kept in the laboratory aquaria and one was sacrificed with the regenerating individuals at each of the preferred stages: 3, 5, 7, and 14 dpe.

2.5. Statistical analysis

Statistical significance of the resulting data was evaluated through ordinary one-way ANOVA that included a Tukey's multiple comparisons test for a single pool variance. All values were reported as the mean ± standard error while a $P < 0.05$ was considered to indicate statistical significance difference between groups. The statistical analyses were performed in GraphPad Prism 6.01.

3. Results

3.1. Characterization of RAR and RXR in the sea cucumber

Contigs baring significant similarity (E value < 10^{-30}) to deuterostome orthologs of RAR and RXR were recognized within a transcriptomic database developed from intestinal and nervous tissue of the sea cucumber *Holothuria glaberrima*. These contigs revealed sequence information from the *UTR* and the open reading frame (ORF) used to determine the complete sequence of each receptor. Primer pairs (Table 1) developed against target regions within these putative sequences were used to amplify (PCR) the cDNA products developed from RNA extractions of normal or regenerating intestinal tissues. These products were subsequently sequenced and analyzed, showing to correspond to the retinoid receptor orthologs of our model system (Fig. 1).

Three RAR isoforms were ultimately characterized and are herein identified as RAR-L (long: 1,314 bp – base pairs / 437 aa – amino acids), RAR-M (midsize: 1,170 bp / 389 aa), and RAR-S (short: 987 bp / 328 aa) in accordance to the length of their respective ORF. All three RAR isoforms are identical at the amino and carboxyl ends, yet a fragment near the middle of the RAR-L isoform is missing from RAR-M (−144 bp) and a larger one from RAR-S (−327 bp – Supplementary data S2). The RAR-M variant displayed the highest overall identity (58%) and similarity (71%) to the ortholog of the sea urchin *Strongylocentrotus purpuratus*, another echinoderm (Table 2). The polypeptide encoded by this variant contains the signature domains characteristic of most all NR: the DNA binding domain (DBD) and the ligand binding domain (LBD – Fig. 2). The highly conserved DBD exhibit 87% identity to the sea urchin's sequence (Table 2) and is completely identical in the other two isoforms (Supplementary data S2). Its sequence features both distinctive C4 type zinc finger motifs coordinating DNA binding and the RAR-RXR heterodimer interface (NRCQ). The LBD within RAR-M incorporates the ligand binding site, co-regulatory recognition site, and heterodimer interface (Fig. 2) which display 57% identity to the sea urchin's ortholog (Table 2). The LBD within the RAR-L variant is 48 aa longer at the beginning of the motif when compared to RAR-M (Supplementary data S2). The addition of these bases occurs without affecting the characteristic functional sites located further

downstream in the domain. On the contrary RAR-S, which is 61 aa smaller than RAR-M, completely eliminates the coregulatory recognition site while partially disrupting the ligand binding site (Fig. 2).

Primer pairs developed against contigs bearing significant similarity to echinoderm orthologs of RXR (Table 1) helped identify and characterize two isoforms within the sea cucumber's transcriptome herein labeled RXR-F (full) and RXR-T (truncated – Fig. 1). The nt sequence for RXR-F is 1,236 bp long; this yields a 411 aa polypeptide (Supplementary data S3) that displays 69% identity and 73% overall similarity to sea urchin's orthologs, *S. purpuratus* and *S. nudus* (Table 2). Its sequence shows the characteristic DBD where both C4 type zinc finger motifs are found alongside a group of heterodimerization interfaces specific for the interaction with NR that include RAR, PPAR, and ecdysone receptor. Its DBD conserves 85% identity to the sea urchin's orthologs which incorporates homodimerization interface types DR1 and DR2; this second interface is disrupted by a small peptide (CVVKFRSAV) after the 3rd aa of this 6 aa motif. Its LBD includes the ligand binding and co-regulatory recognition sites in addition to the homodimerization and heterodimerization interfaces characteristic of the region (Fig. 3). The sequence of RXR-T is almost identical to RXR-F; yet the absence of 26 bp towards the end of its DBD translates for a truncated protein of 199 aa that shifts the reading frame by +1 nt. This frame shift produces a stop signal that removes all but the first 22 aa of the LBD, consequently eliminating the characteristic functional motifs of the domain (Supplementary data S3).

Analysis of the sequencing data revealed three somewhat different 5′ *UTR* associated to RAR and one for RXR herein identified as RAR-5′UTR-A (394 bp), RAR-5′*UTR*-B1 (1,041 bp), RAR-5′*UTR*-B2 (471 bp), and RXR-5′*UTR* (243 bp – Supplementary data S4). Meanwhile, one *UTR* was recognized along the 3′ end of each receptor and labeled RAR-3′*UTR* (61 bp) and RXR-3′*UTR* (655 bp – Supplementary data S5). Sequencing data revealed a 178 bp segment located directly upstream of the transcription start site which is identical in all three RAR-5′*UTR*. As we analyzed further upstream of this shared region we detected that RAR-5′*UTR*-A completely diverges from the sequences of both RAR-5′*UTR*-B variants (−B1, −B2). The −B variants share a total of 471 bases, including the 178 bases common to all three 5′ leader sequences, nonetheless a fragment of 570 bases absent in the −B2 subtype can be distinguished after the ninety-eighth base of the −B1 variant. Scanning for regulatory RNA motifs within these 5′*UTR* revealed the presence of upstream open reading frames (uORF) and upstream initiation codon (uAUG) along each holothurian retinoid receptor isoform (Supplementary data S4). A similar analysis along the 3′*UTR* revealed the characteristic polyadenylation signal within RAR (Supplementary data S5).

3.2. Phylogenetic analysis of the sea cucumber's RAR and RXR display ambulacrian ancestry

A maximum likelihood phylogenetic tree was prepared from a multiple sequence alignment including the sea cucumber's variants, RAR-M and RXR-F, probed against orthologs from different metazoans phylogenies and sequences from other nuclear receptors to serve as outliers for the analysis (Fig. 4). At first glance we notice that all retinoic acid receptors cluster at the top of the phylogram and all retinoid X receptors cluster at the bottom of it. In

the middle of these two groups lay the four *Strongylocentrotus purpuratus* nuclear receptors included in the analysis (thyroid hormone receptor, ecdysone receptor, estrogen related receptor, and peroxisome proliferator-activated receptor) separating each retinoid receptor clade. Upon closer examination, it can be observed that both retinoid receptors identified in the sea cucumber cluster within the ambulacrian branch in each clade, grouping alongside acorn worms (hemichordata) and sea urchins (echinoids); all basal deuterostomes. In both instances the ambulacrian clade is flanked by protostomes (e.g. lophotrochozoa and/or ecdysozoa) among the neighboring branches towards the middle of the tree and by non-vertebrate chordates such as tunicates and cephalochordates along the branches at the other side. Further towards the opposing ends of the tree we distinguish that vertebrate phylogenies are precisely distributed among the alpha, beta, and gamma branches depicting the paralog groups characteristic of the lineage (Fig. 4).

3.3. Expression pattern of the retinoid receptors in various visceral organs

The bioinformatics analyses shown above suggest that three different isoforms of RAR and two of RXR are expressed along the intestinal tissue. Nonetheless, it remains to be demonstrated if these sequences are expressed in other visceral organs. Semiquantitative reverse transcription PCR analysis revealed that all retinoid receptors are expressed in each of the analyzed tissues; including the mesenteries, esophagus, respiratory trees, gonads, muscles, descending small intestine, the region connecting the ascending small intestine to the large intestine, and the large intestine (Fig. 5). Specifically, we detected a higher expression of RAR-L in the anterior intestinal tissue and respiratory trees when compared to the gonads (Fig. 5a). A similar observation was noticed with the RAR-M variant, showing it was highly expressed in the anterior intestinal tissue, respiratory trees, and esophagus when compared to the gonads (Fig. 5b). The third RAR variant, RAR-S, and both RXR variants (RXR-F and RXR-T) were similarly expressed among the studied tissues (Fig. 5 c, d, e).

3.4. Expression pattern of the retinoid receptors in normal and regenerating intestinal tissue

We further explored if the expression patterns of the retinoid receptor isoforms underwent changes during the regenerative processes of the gut. Semiquantitative PCRs were completed employing cDNA from the anterior, medial, and posterior segments of the regenerating intestine at four different regenerative stages (3, 5, 7, and 14 dpe) and in normal individuals. Analysis of the resulting data shows that RAR-L and RXR-F are differentially expressed between the studied stages at the posterior end of the intestinal tissue while RAR-S is differentially expressed at its anterior end (Fig. 6). The results show that the expression of RAR-L increases during the early regenerative stages and peaks at the 7 dpe stage (Fig. 6c). Meanwhile the expression of RAR-S is similar to the normal values during the first week of regeneration and significantly drops by 14 dpe (Fig. 6g). On the other hand, the expression of RXR-S decreases during the first regenerative stage, significantly drops by 5 dpe, but then increases at 14 dpe (Fig. 6l).

4. Discussion

In the current study we identified and characterized the sequences of RAR and RXR expressed by the sea cucumber, *H. glaberrima* from EST and RNA-seq databases. Contigs within these databanks revealed a putative sequence for each retinoid receptor. Each sequence was later used to design primers to validate their full-length sequence following RNA extraction, cDNA amplification, and sequencing. Sequencing data analysis clearly establishes that these correspond to the holothurian homologs of the retinoid receptors – RAR and RXR. The supporting evidence shows that: (1) Our sequences exhibit the A-F structural scheme characteristic of the NR superfamily of ligand modulated transcription factors (Green and Chambon, 1988). (2) They display the two distinctive motifs characteristic of the superfamily, the DNA binding domain (DBD) and the ligand binding domain (LBD), respectively allocated along the C and E structural segments of each receptor (Robinson-Rechavi et al., 2003). (3) The aa comprising the DBD in the holothurian receptors, preserve the characteristic structural organization around the cysteine residues known to be important for the structural coordination of the zinc finger motifs – Cys-X2-Cys-X13-Cys-X2-Cys-X15-Cys-X5-Cys-X9-Cys-X2-Cys (Nakata, 1995; Rastinejad et al., 1995). (4) The LBD of each receptor incorporates distinctive regions such as the ligand binding pocket and the transactivation domain (Evans and Mangelsdorf, 2014) despite the relative variability displayed by the superfamily along this region (Kostrouchova and Kostrouch, 2014).

Sequencing data also revealed the expression of different isoforms of RAR and RXR within the transcriptome of this holothurian. Indeed, the presence of multiple isoforms of each retinoid receptor has been documented across most metazoans (Gutierrez-Mazariegos et al., 2014). Between two and four isoforms of each retinoid receptor gene have been documented in both vertebrates and invertebrates, including other echinoderms, as a consequence of differential promoter usage and alternative splicing (Zelent et al., 1991; Zimmer et al., 1994). The results depicted herein harmonize with what is known, as we were able to identify three RAR and two RXR isoforms; all of which appear to be the product of alternative splicing of a single RAR and a single RXR gene coding the different isoforms.

We identified one isoform of each retinoid receptor displaying an irregular structural scheme that affects their LBD. Specifically, the splicing events characteristic of the RAR-S isoform eliminates the coregulatory recognition site and partially disrupts the ligand binding domain; meanwhile splicing of RXR-T eliminates the LBD altogether. The expression of unorthodox NR exposing structural variability along the LBD is not an uncommon incident. In fact, it has been reported that members of the NR0A subfamily do not express the LBD at all (Zhang et al., 2004; Thomson et al., 2009). Further studies report the existence of unconventional NR in which specific functional regions within the LBD, such as the ligand binding pocket or the transactivation domain, are modified or not present (Benoit et al., 2006). Interestingly, there is strong evidence supporting that these and other NR exposing structural variability along the LBD have functional roles in different organisms. For example, nematodes express a nonconserved NR variant lacking the LBD (e.g. ODR-7/NR0A4) (Kostrouchova and Kostrouch, 2014) which promotes differentiation of ciliated neurons along their highly developed chemosensing system (Sengupta et al., 1994).

Structural and functional analyses of NR in murine cell cultures expose a different variety of ligand-independent NR (e.g. Nurr1/NR4A2) which displays several hydrophobic residues along the ligand binding pocket's cleft that proves restrictive to fit a ligand. Signaling through this unconventional NR, which also lack the classical AF-2 transactivation site (Wang et al., 2003), is induced by forming heterodimers with RXR (Wallén-Mackenzie et al., 2003).

The paralogs of each retinoid receptor and their isoforms have been found to exhibit spatially and temporally restricted expression patterns depending on the context in which they are translated (Campo-Paysaa et al., 2008). This is particularly true during development as has been documented in fish, amphibian and mammalian development (Dollé, 2009, Linville et al., 2009). However, some receptors have been found to be expressed ubiquitously; for example RARα, RXRα, and RXRβ displayed ubiquitous expression during development of the murine digestive tract (Dollé, 2009). Here we showed that the retinoid receptors identified in our model system are expressed in all studied tissues. Specifically, RAR-L and RAR-M were highly expressed in the intestine (anterior), esophagus, and/or the respiratory trees when compared to the gonads; meanwhile RAR-S, RXR-F, or RXR-T exhibited a similar expression rate along the organs tested.

Specific retinoid receptors variants have also been linked with the regenerative events in different organisms. For example, RARα, RARβ, and RXRα are all elevated in the nerve distal to the injury site during nerve regeneration following peripheral crush injury in rats (Zhelyaznik and Mey, 2006). Further studies reported that RARγ is the highest expressed isoform across the blastemal tissue during caudal fin regeneration in the zebrafish (White et al., 1994); meanwhile RARβ2 is regarded as an essential receptor mediating retinoid induced tail regeneration in adult newts (Carter et al., 2011). In other circumstances RARα and RXRβ showed specific expression patterns across the skin, perichondrium, and mesenchyme during deer antler regeneration (Allen et al., 2002). Analysis of the expression pattern of the holothurian retinoid receptor isoforms during intestinal regeneration showed that RAR-S is differentially expressed between the 5 and 14 dpe stages at the anterior intestinal tissue. Meanwhile RAR-L and RXR-F are differentially expressed along the posterior intestinal tissue between the 3 and 7 dpe or the 5 and 14 dpe stages, respectively. However, in contrast to regenerative models in which the difference in expression between the various retinoid receptors is remarkable (e.g. limb or fin), the expression during the various stages of intestinal regeneration in the sea cucumber, although significant, does not appear to be as striking; for both RAR and the RXR isoforms appear to be continuously expressed, if at different levels. There are various possible explanations for this unexpected lack of specific expression during intestinal regeneration. Among these: that (1) differential expression is occurring at the level of the various cell or tissues in the intestine, but cannot be detected at the organ level, (2) changes in levels of expression are indeed occurring but are small enough that cannot be fully detected with semiquantitative reverse transcription PCR analysis, (3) effects of other regulatory elements that can influence gene expression at the level of translation such as uORFs (4) retinoid receptor specificity does not play a significant role in visceral regeneration the way they might play a role in the regeneration of limbs and other external structures. Finally, it is important to highlight that these receptors are but one of the players in the retinoic acid signaling pathway and that other mediators,

such as synthesis or degradation, might be playing an even greater influence on the activity of the RA signaling pathway.

In conclusion, we identified and characterized the sequences of both retinoid receptors and their isoforms in the echinoderm *H. glaberrima.* Our results established the basal expression of these receptors' transcripts among the visceral organs in normal individuals or during the regenerative events of the gut. We also suggest that the expression of these receptors could be regulated during events of translation. This information represents the first step towards a more detailed understanding of the relationship between these genetic sequences and the regenerative prowess exhibited by this model system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

aa	amino acid(s)
bp	base pair(s)
cDNA	DNA complementary to RNA
dpe	days post evisceration
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
EST	expressed sequence tags
NR	nuclear receptor(s)
ORF	open reading frame
RA	retinoic acid
RAR	retinoic acid receptor(s)
RXR	retinoid X receptor(s)
PCR	polymerase chain reaction
UTR	untranslated region(s)
uAUG	upstream initiation codon(s)
uORF	upstream open reading frame(s)

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Highlights

- Identified and characterized retinoid receptors in a sea cucumber.
- Sequencing data revealed the presence of isoforms for each receptor.
- Established basal expression of these among visceral organs or regenerating gut.

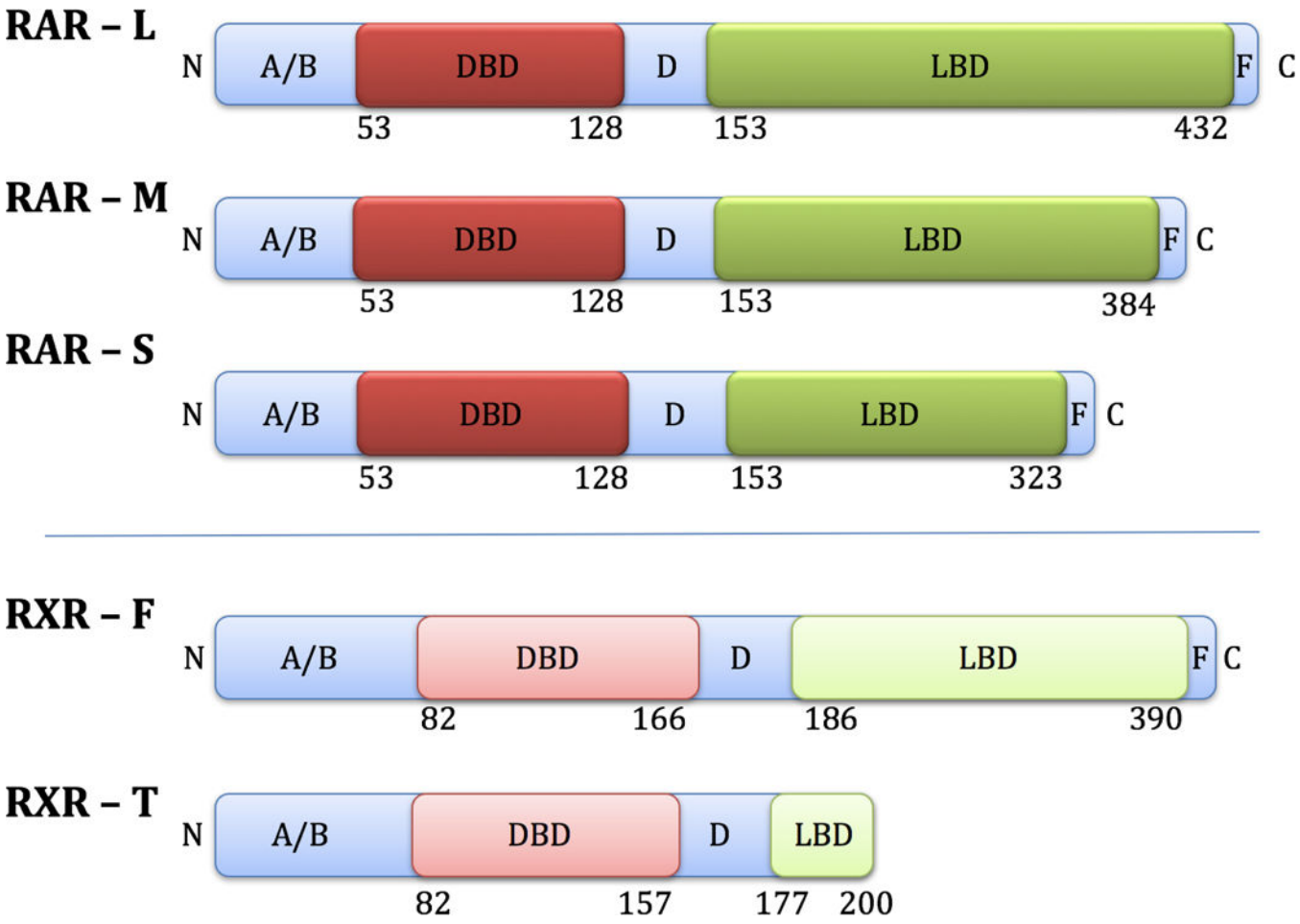


Fig. 1.

Diagram of the A-F structural scheme for the predicted retinoid receptors proteins identified in *H. glaberrima.* (COLOR FIGURE)

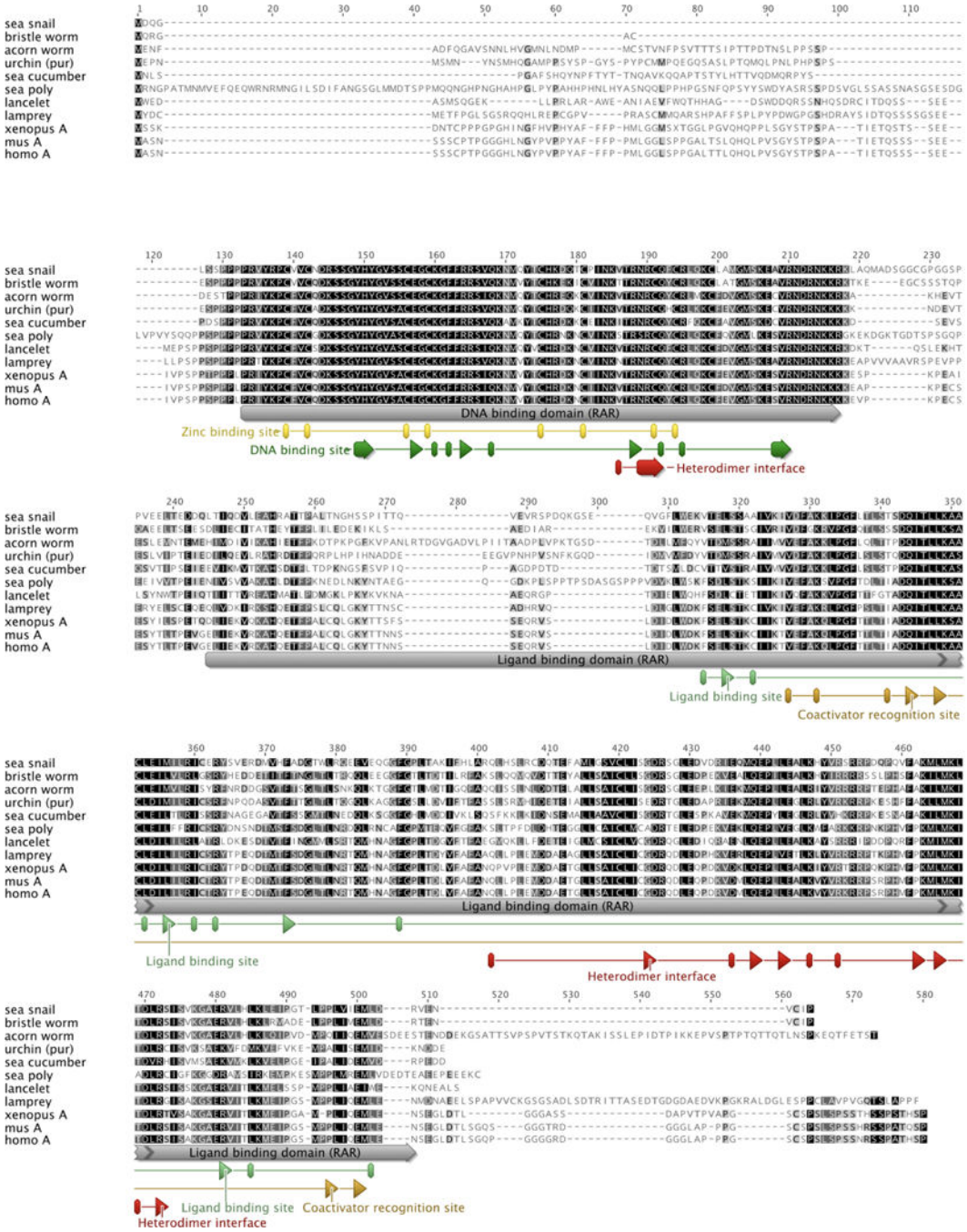


Fig. 2.

Multiple sequence alignment of the sea cucumber RAR-M amino acid sequence and those of selected protostomes, deuterostomes, chordates, and vertebrates. Conserved residues are shaded in black when they are 100% similar, dark grey if they are 99–80% similar, light grey when 79–60% similar, or white when less than 60% similar. The characteristic functional domains, sites, and interfaces of the protein are depicted herein. (COLOR FIGURE)

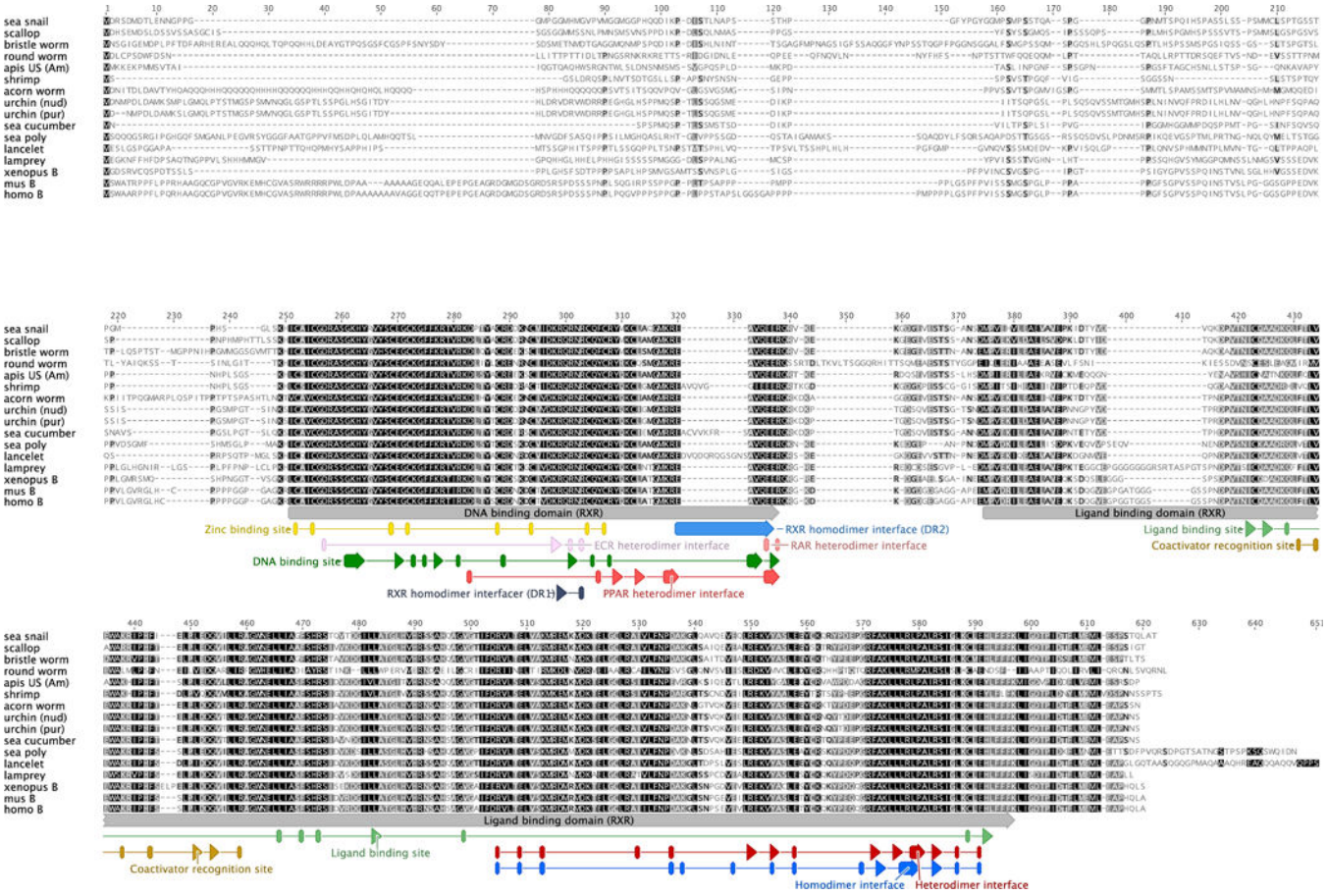


Fig. 3.

Multiple sequence alignment of the sea cucumber RXR-F amino acid sequence and those of selected protostomes, deuterostomes, chordates, and vertebrates. Conserved residues are shaded in black when they are 100% similar, dark grey if they are 99–80% similar, light grey when 79–60% similar, or white when less than 60% similar. The characteristic functional domains, sites, and interfaces of the protein are depicted herein. (COLOR FIGURE)

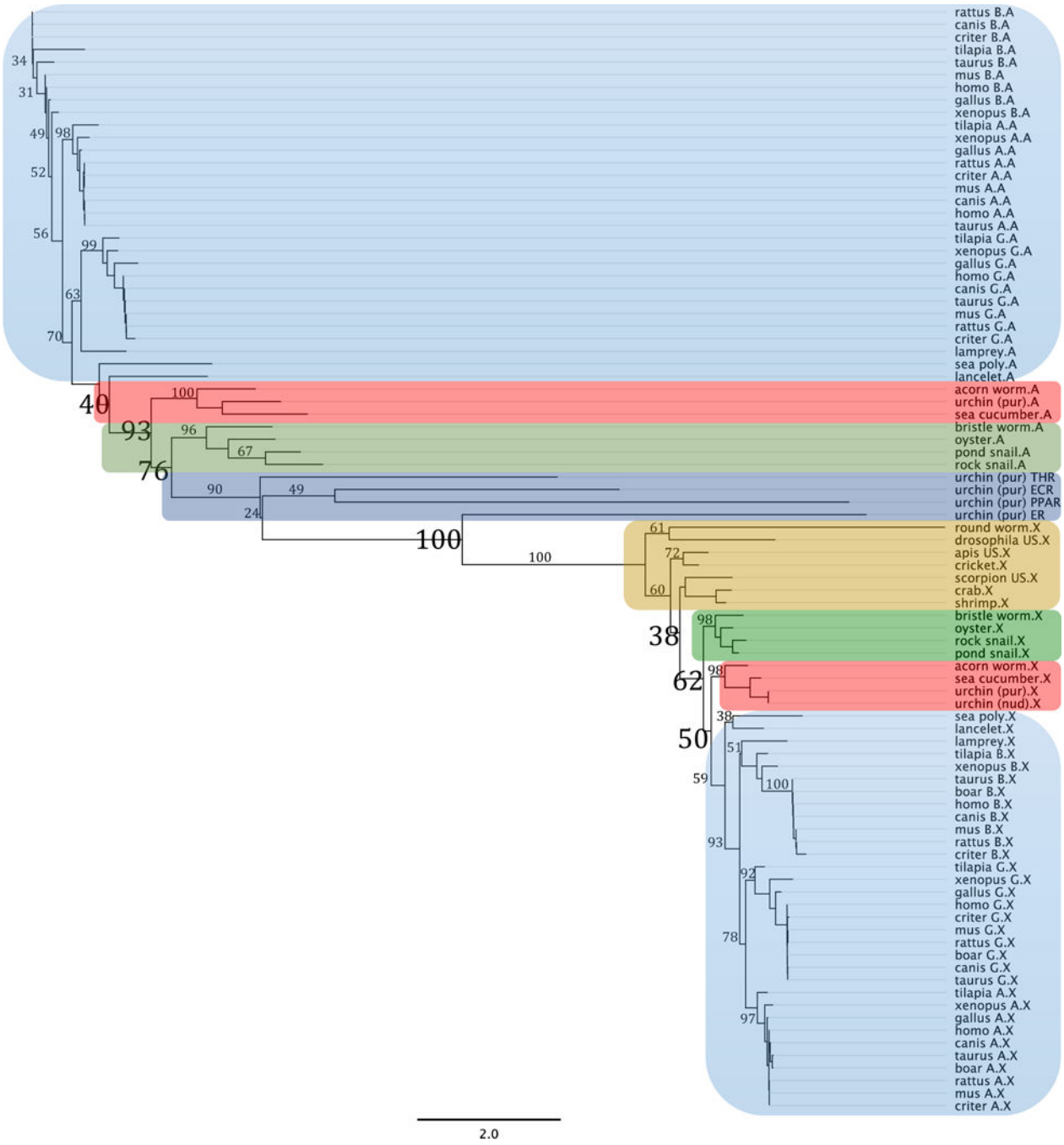


Fig. 4.

Maximum likelihood phylogram based on the amino acid sequences of the retinoid receptors found in the sea cucumber, *Holothuria glaberrima*, compared against its homologs in multiple species and other nuclear receptors from the sea urchin. Every retinoic acid receptor localizes to the top of the phylogram and is identified by a .A suffix, while every retinoid X receptor localize to the bottom of it and is identified by the .X suffix. Separating both retinoid receptor clades lay the four nuclear receptors included in the analysis. Vertebrates within the chordate taxon were distributed among the alpha, beta, and gamma branches

depicting the paralogs characteristic of this lineage. Every sequence used for this analysis is referenced in Supplementary data S1. Major taxonomic groups are defined by color boxes (Chordates = blue, Non-chordate deuterostomes = red, Lophotrochozoa = green, Ecdysozoa = yellow, and other NR = grey) and by the bootstrap values depicted in a larger font. The numbers at the nodes correspond to the bootstrap proportion expressed as the percentage of 1000 replicates while the length of the branches is proportional to the amount of inferred evolutionary change as defined by the bar in substitutions per site. COLOR FIGURE

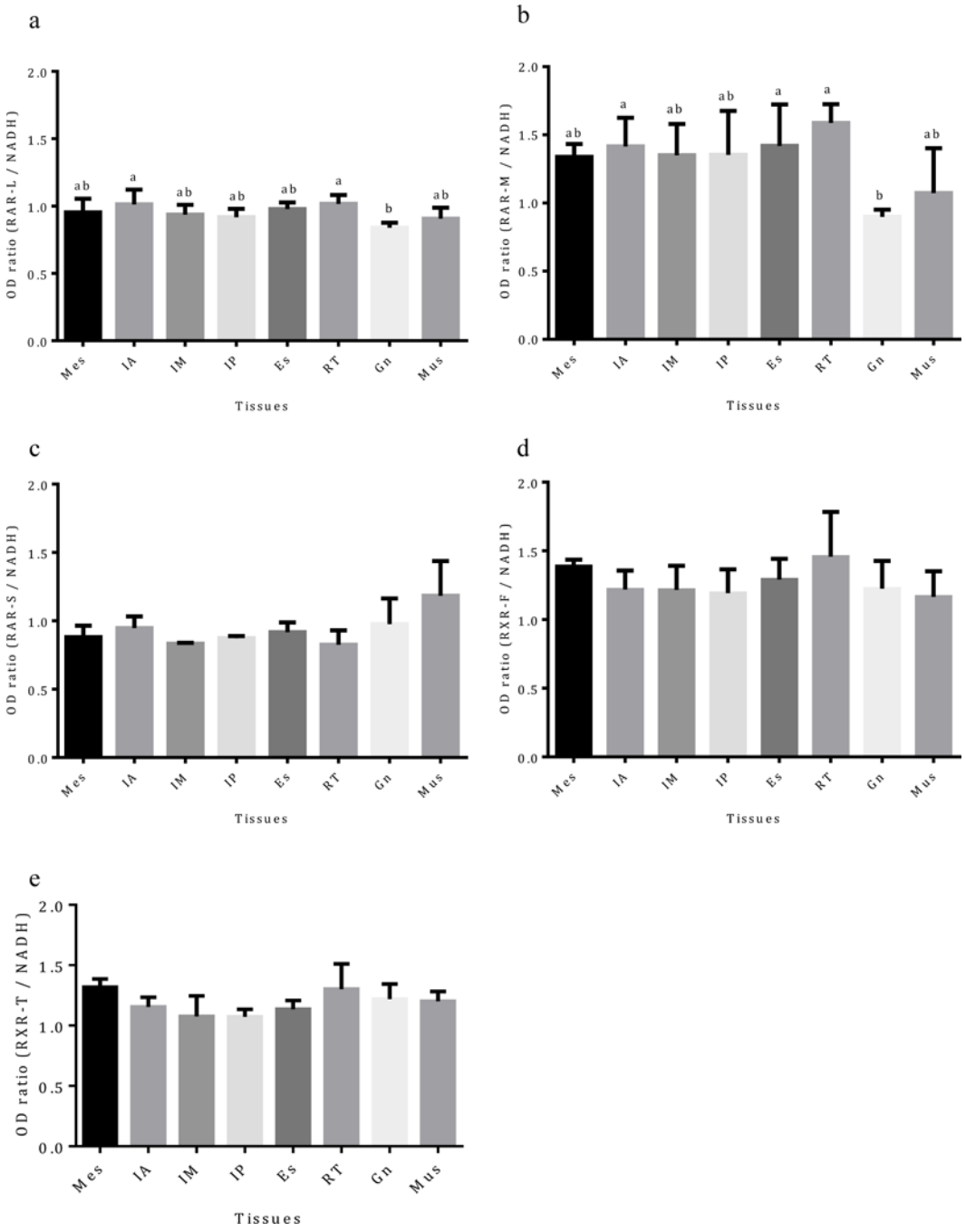
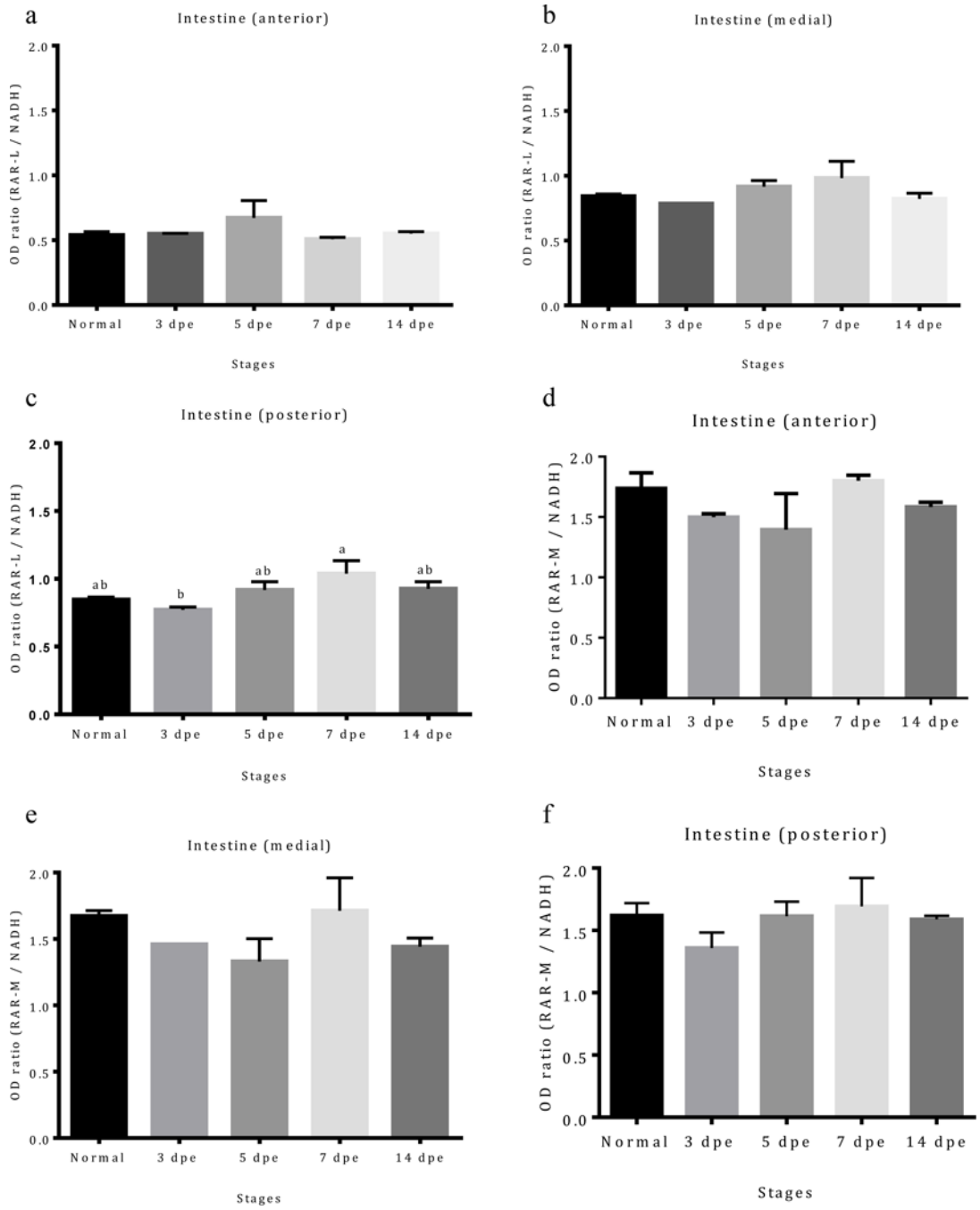
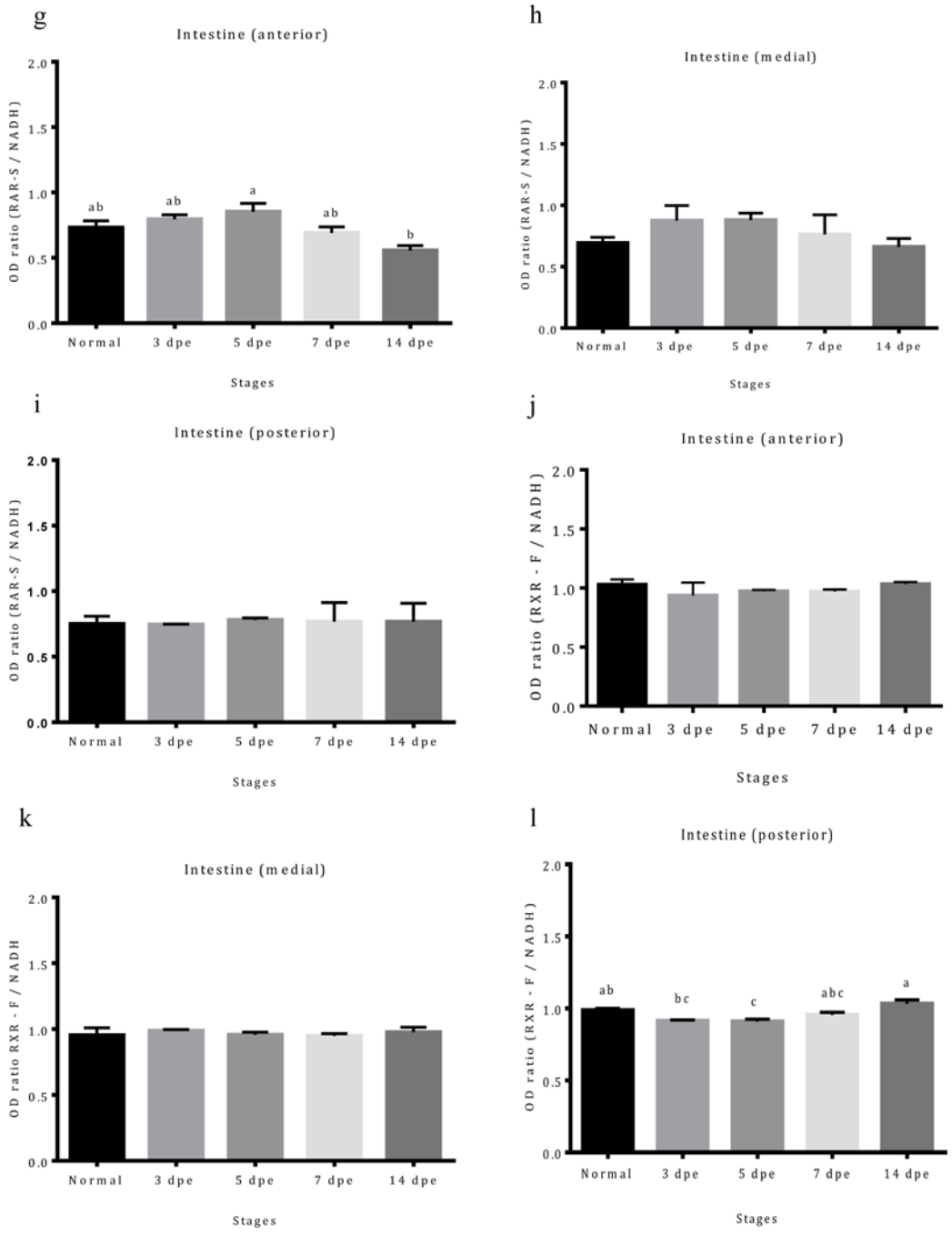


Fig. 5.

mRNA expression profiles of retinoic acid receptor and retinoid X receptor along different tissues of the adult sea cucumber. At least three biological replicates were performed per group: mesentery (Mes) n=3, anterior intestinal tissue (IA) n=6, medial intestinal tissue (IM) n=6, posterior intestinal tissue (IP) n=6, esophagus (Es) n=7, respiratory tree (RT) n=3, gonads (Gn) n=5, and muscle (Muc) n=3. Ordinary one way ANOVA showed differences among means statistically significant in graphs a (P=0.02) and b (P=0.008). A multiple comparison test (Tukey) in these two graphs (a and b) showed significant difference

($P<0.05$) between groups bearing different letters, while groups bearing both letters are not statistically different to those with either letter.





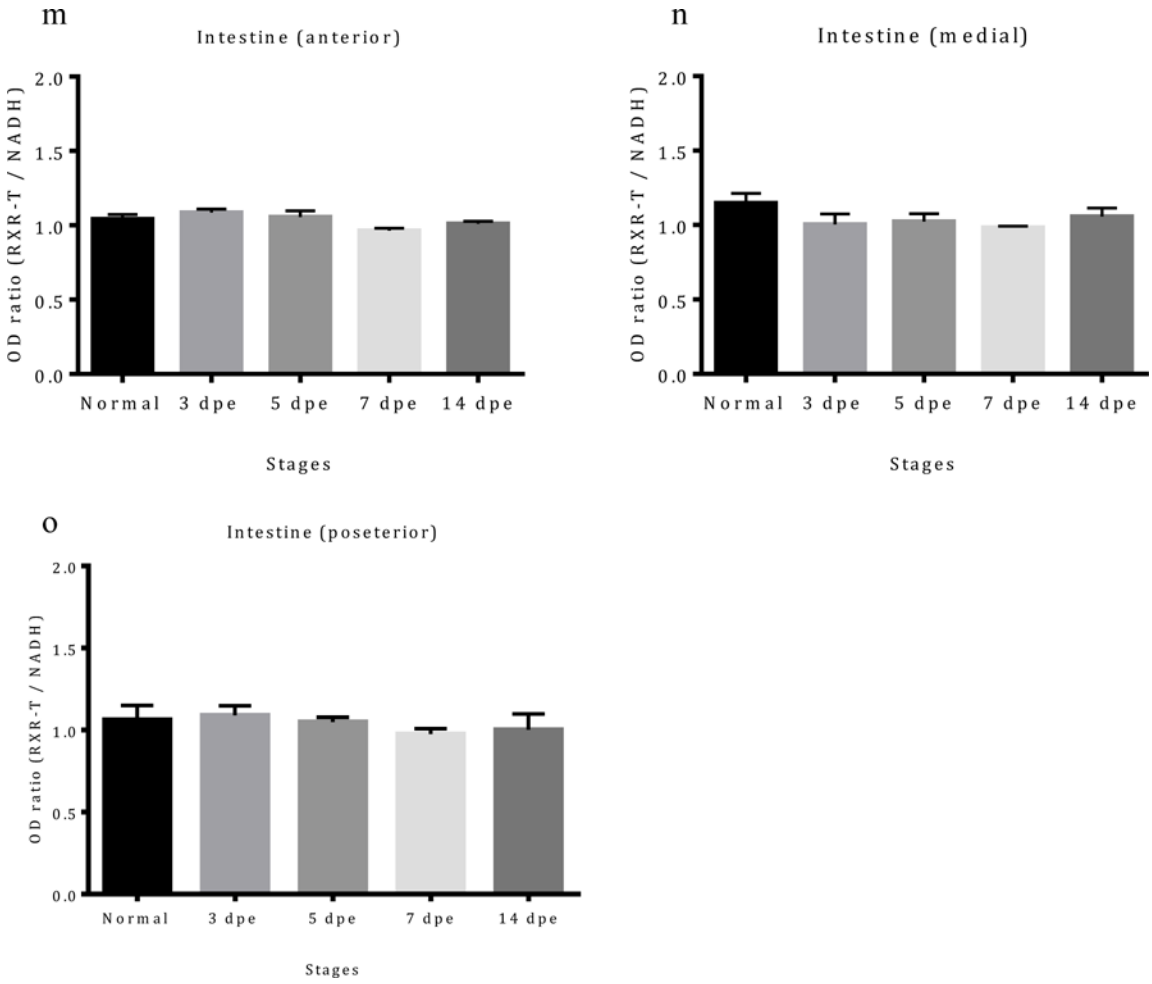


Fig. 6.

mRNA expression profiles of retinoic acid receptor and retinoid X receptor during different regenerative stages (3, 5, 7, and 14 days post evisceration) and in normal intestinal tissue. Three biological replicates were analyzed at each regenerating interval and four in the normal gut. Ordinary one way ANOVA showed differences among means statistically significant in graphs c ($P=0.04$), g ($P=0.02$), and l ($P=0.002$). A multiple comparison test (Tukey) showed significant difference ($P<0.05$ or $P<0.01$) between groups bearing different letters in these three graphs. Those groups bearing two or three letters are not statistically different to those with any of the letters.

Table 1

PCR primers employed in the current study. Primers labeled with an asterisk (*) were used during the semi-quantitative PCR analyses.

Retinoic acid receptor		
Primer name	**Sequence (5′ - 3′)**	**Amplicon product region**
RAR 5AF1	CAAGTTTGAATCGTTGTGGGAAAG	5′UTR A variant
RAR 5BF1	CACACAGGTTCATTCAGACAAGTTC	5′UTR B1 variant
RAR 5BF2	TAACACCACCAGCAAAGTCAGA	5′UTR B1 variant
RAR 5BR2	TGACTTTGCTGGTGGTGTTAA	5′UTR B1 variant
RAR 5BF3	GTATGTTGGAGGAGGCAGTTTTG	5′UTR both B variants
RAR 5BR1	CAAAACTGCCTCCTCCAACATAC	5′UTR both B variants
RAR 5CR1	GATAGGGAGATGCTACTGGAGATC	5′UTR all variants
RAR 5CR2	AATCCACCTTCCCACTACTTCC	5′UTR all variants
RAR 5′F	TCGGAAGTAGTGGGAAGGTGGATT	5′UTR all variants
RAR 5CR3	AAAGGCACCTGGACTGAGG	ORF all variants
RAR ORF	GCCATGAAGTACACATGCCACA	ORF all variants
RAR ORF3*	AAAGCGCACAGCGATACCTTCCT	ORF all variants
RAR 5′R	AGGAAGGTATCGCTGTGCGCTTT	ORF all variants
RAR FC1	TCTCAGTTACCTTGAGGATAAGCTCACG	ORF RAR-S variant
RAR RC2*	ACCGTGAGCTTATCCTCAAGGTAACTG	ORF RAR- S variant
RAR RC1	CGTGAGCTTATCCTCAAGGTAACTGAGA	ORF RAR-S variant
RAR FRT3	CTGTGTGACAACAGTATCCACAAGAGC	ORF RAR-M variant
RAR RRT2	GCTCTTGTGGATACTGTTGTCACACAG	ORF RAR-M variant
RAR ORF2	ATCCGCCACCTGCTGTTAATTTGC	ORF RAR-L variant
RAR FC1	TCTCAGTTACCTTGAGGATAAGCTCACG	ORF all variants
RAR ORR4*	TCCTTCACCAGCATTGAACCGTGA	ORF all variants
RAR RRT	TTCAAGACCTGTTCGATCTCCGCT	ORF all variants
RAR ORR	TGCTCTCCTTTGGTCTCCGTTTGT	ORF all variants
RAR 3′F	TGTCCACAAACGGAGACCAAAGGA	ORF all variants
RAR 3′R	TGCAGCATGATTCCCGCATCAAAG	3′UTR
Retinoid X receptor		
Primer name	Sequence (5′ - 3′)	Amplicon product region
RXR 5AF1	TTTATGATCTGTGGCTGGGCAT	5′UTR
RXR 5′F	ATTGGACCATCCTCCGTCAGTA	5′UTR
RXR ORF	ATCAACTTCTCCCAGGTCAGCCAA	ORF both variants
RXR ORR	AGTTCATTCCAGCCTGCTCTGAGT	ORF both variants
RXR ORF2	TGCCGCTATCAGAAGTGCATTGAG	ORF both variants
RXR F2SEQ*	GGATGAGGAGAGAAGCCTGTGTTGTTA	ORF RXR-T variant
RXR F2SEQ-B*	GGATGAGGAGAGAAGGTACAAGAAGAG	ORF RXR-F variant
RXR ORR2	TGGTTCCACTGCTAACTCTGCCTC	ORF both variants
RXR ORR2-1*	TGGTTCCACTGCTAACTCTGCCT	ORF both variants
RXR 3′F	CAGGTTACTTTACTCAGAGCAGGC	ORF both variants

Retinoic acid receptor		
Primer name	**Sequence (5′ - 3′)**	**Amplicon product region**
RXR 3′R	ACGGTGGCTCCTCTTGTGCAATAA	3′UTR
RXR 3′F2	CACAAGAGGAGCCACCGTA	3′UTR
RXR 3′R2	GAAGCCATCACCTGAAGATG	3′UTR
NADH		
Primer name	Sequence (5′ - 3′)	Amplicon product region
NADH F*	CGCAGAAGTAGCCGCGAATAT	semiQ-PCR ctrl
NADH R*	CAATGGTTGTTGCTGGAGTCTTT	semiQ-PCR ctrl

Table 2

The overall identity (similarity) amid the deduced amino acid sequence of *Holothuria glaberrima* retinoid receptors and the ortholog receptors in other organisms. All values are presented in percentages. (n.a. = does not apply)

	Retinoic acid receptor			Retinoid X receptor		
Organism's nickname	**Complete protein**	**DBD**	**LBD**	**Complete protein**	**DBD**	**LBD**
sea snail	45 (59)	78 (91)	46 (63)	62 (70)	73 (83)	86 (92)
scallop	n.a.	n.a.	n.a.	62 (70)	79 (87)	85 (94)
bristle worm	47 (63)	82 (88)	45 (69)	51 (58)	76 (86)	86 (92)
round worm	n.a.	n.a.	n.a.	33 (48)	77 (85)	38 (58)
apis US (Am)	n.a.	n.a.	n.a.	55 (66)	77 (88)	69 (83)
shrimp	n.a.	n.a.	n.a.	60 (71)	75 (88)	77 (85)
acorn worm	53 (68)	84 (94)	54 (69)	61 (66)	83 (87)	94 (97)
urchin (nud)	n.a.	n.a.	n.a.	69 (73)	86 (89)	95 (96)
urchin (pur)	58 (71)	87 (94)	57 (73)	69 (73)	85 (89)	95 (96)
sea poly	35 (49)	81 (89)	36 (58)	48 (56)	79 (87)	76 (86)
lancelet	41 (55)	80 (91)	39 (59)	56 (62)	76 (84)	85 (91)
lamprey	43 (57)	86 (89)	45 (66)	57 (64)	80 (86)	76 (85)
xenopus	44 (59)	87 (92)	43 (66)	58 (66)	76 (86)	78 (88)
mus	46 (60)	87 (92)	45 (65)	51 (59)	75 (85)	78 (88)
homo	46 (60)	87 (92)	45 (65)	50 (57)	75 (85)	78 (88)