Three orphan nuclear receptors in the scleractinian coral *Pocillopora damicornis* from the Pacific coast of Costa Rica

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Abstract: Within the animal kingdom, nuclear receptors modulate transcription to regulate diverse processes including embryonic development, energetic homeostasis and sexual differentiation. To date, nuclear receptor sequences have been reported in only a few cnidarian species, and little is known about nuclear receptor function in cnidarians. We have identified three nuclear receptor genes expressed in *Pocillopora damicornis* coral colonies (PdNR1, PdNR6, and PdNR7). The cDNA and deduced amino acid sequences indicate that these three genes are closely related to nuclear receptors previously reported in *Acropora millepora* planula larvae (AmNR1, AmNR6, and AmNR7). None of the three *P. damicornis* nuclear receptors are apparent homologs of known ligand-activated receptors. Two are most closely related to vertebrate genes important in neural differentiation and signaling (subfamily 2E, tailless genes and subfamily 2F, COUP-TFs). The third cannot be unambiguously classified within a currently recognized subfamily. Future studies are needed to describe the roles of nuclear receptors in cnidarian development and physiology. Rev. Biol. Trop. 56 (Suppl. 4): 39-48. Epub 2009 June 30.

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Nuclear receptors are a superfamily of transcription factors that are conserved throughout the animal kingdom and regulate diverse aspects of development, differentiation, metabolism, reproduction, and other physiological processes (Mangelsdorf *et al.* 1995, Escriva *et al.* 1997, Maglich *et al.* 2001, Gissendanner *et al.* 2004). Nuclear receptor proteins share a common structural organization that includes a highly conserved DNA-binding domain and a moderately conserved ligand-binding domain (Fig. 1. Reviewed by Chawla *et al.* 2001, Handschin & Meyer 2003, Bain *et al.* 2007). In both protostomes and deuterostomes, some nuclear receptors are activated by small lipophilic ligands; thus they serve as receptors for endogenous hormones (e.g. steroids, retinoids, and thyroid hormones), dietary metabolites and other compounds (Laudet *et al.* 1992, Beato *et al.* 1995, Mangelsdorf & Evans 1995, Thummel 1995, Laudet 1997, Blumberg & Evans 1998). Environmental contaminants can also bind to nuclear receptors in place of a natural ligand, disrupting endogenous signaling pathways (Tyler *et al.* 1998, deFur *et al.* 1999, McLachlan 2001, Oberdorster & Cheek 2001, Cheek 2006). Other nuclear receptors, the "orphan" receptors, lack known ligands. While

Fig. 1. Schematic representation of functional domains of a generic nuclear receptor (adapted from Handschin and Meyer 2003). From the amino terminus, nuclear receptors are divided into domains that include: (1) A highly variable transcriptional activation (AF-1) domain; (2) a conserved DNA-binding domain (DBD) comprised of two zinc finger motifs that bind to response elements in the promoters of target genes; (3) a variable hinge region that increases protein flexibility to allow for protein:protein (e.g. dimerization) and protein:DNA interactions: (4) the carboxy terminus that includes ligand-binding, dimerization and activation functions (AF-2). This entire carboxy terminus is collectively referred to as the ligand-binding domain (LBD) throughout this manuscript (e.g. Fig. 2).

ligands may be identified for some orphan receptors in the future, others may be activated through alternative mechanisms or possess constitutive activity (i.e. are inherently active) (Giguere *et al.* 1988, Power *et al.* 1991, Marcus *et al.* 1996, Escriva *et al.* 1997).

Nuclear receptors are a key component of the endocrine system, and their roles as hormone receptors have been particularly well-characterized in vertebrates and insects. In contrast to these highly organized and compartmentalized animals, cnidarians have a "tissue grade" organization (i.e. have specialized tissues that are not further organized into organs or systems) and circulation occurs primarily by diffusion. Intercellular signaling processes in cnidarians are likely to be largely paracrine (i.e. cells secrete a hormone that affects nearby cells) rather than endocrine (i.e. cells secrete a hormone that travels through the circulatory system to affect targets cells that may be distant). While progress has been made toward understanding signaling processes in cnidarians (reviewed by Leitz 2001, Grimmelikhuijzen *et al.* 2002, Müller y Leitz 2002, Tarrant 2005, 2007), it is not currently known whether any nuclear receptors transduce the signals of endogenous cnidarian hormones.

In cnidarians, nuclear receptor genes have initially been identified using reverse transcription and the polymerase chain reaction (RT-PCR) with degenerate primers. Cnidarian nuclear receptors identified through these methods include one from the hydra *Hydra vulgaris* (Escriva *et al.* 1997), one from the jellyfish *Tripedalia cystophora* (Kostrouch *et al.* 1998), four from the anemone *Anemonia sulcata* (Escriva *et al.* 1997), one from the scleractinian coral *Montipora capitata* (GenBank accession number AF254813) and ten from the scleractinian coral *Acropora millepora* (Grasso *et al.* 2001). Sequencing of the genome of the sea anemone *Nematostella vectensis* (Sullivan *et al.* 2006) has provided a recent opportunity to survey cnidarian nuclear receptor diversity on a genome-wide scale. Preliminary analyses suggest that nuclear receptor diversity in *N. vectensis* is comparable to that described in *A. millepora* (A. Reitzel, pers. comm.; unpubl. data). Among the cnidarian nuclear receptors identified to date, most can be placed within family 2, and some of these genes have clear vertebrate homologs, including "tailless" genes (subfamily 2E, Grasso *et al.* 2001), COUP-TF (chicken ovalbumin upstream promoter transcription factor, subfamily 2F, Escriva *et al.* 1997, Grasso *et al.* 2001) and RXR (retinoid "x" receptor, subfamily 2B, Kostrouch *et al.* 1998). Other cnidarian genes are more divergent and are not obvious homologs of genes described in other taxa (Grasso *et al.* 2001).

In corals, nuclear receptors are likely to regulate aspects of reproduction, development and homeostasis, as they do in bilaterian animals. Growth and reproduction of scleractinian corals are essential to the persistence of coral reefs and to the recovery of damaged reef ecosystems. Thus, if nuclear receptors regulate coral physiology, then understanding nuclear receptor signaling in corals will provide important insights into coral health. Coral reefs and coral communities often occur in close proximity to human population centers, and reef organisms may be exposed to an array of

chemical pollutants. It is not currently known whether environmental contaminants can disrupt nuclear receptor signaling in corals or how such disruption would affect coral physiology. In this study, we have identified three nuclear receptor genes in the coral *Pocillopora damicornis,* a scleractinian coral common on shallow reefs throughout the Pacific. This study provides a basis for future functional characterization of coral nuclear receptor signaling and signal disruption.

MATERIALS AND METHODS

Sample Collection: divers collected *P. damicornis* fragments (<5 g) from shallow (1-5 m) patch reefs in Bahía Culebra (Golfo de Papagayo, Costa Rica) and Kaneohe Bay (Oahu, Hawaii). Samples were preserved in RNAlater (Ambion).

RNA Extraction and cDNA Synthesis: coral fragments were pulverized in liquid nitrogen using a mortar and pestle. Pulverized coral tissue was added to a glass homogenizer tube and ground with a teflon-coated homogenizer in ten volumes of a lysis buffer, according to one of two methods. The RNA used in initial studies (cloning nuclear receptors with degenerate primers) was extracted using the acidic guanidine thiocyanate-phenol-chloroform method (Chomczynski & Sacchi 1987). RNA quality was confirmed by optical density (260/280 ratio) and visualization on a denaturing agarose gel. Complementary DNA (cDNA) was synthesized from total RNA (2 µg/20 µl synthesis reaction) using the Omniscript cDNA synthesis kit (Qiagen) with random hexamers.

In subsequent studies, (i.e. to produce the RNA used in RACE reactions), total RNA was extracted with RNA STAT-60 (Tel-Test, Inc.) and treated with DNase (Turbo DNase, Ambion). The DNase-treated total RNA was reprecipitated and subsequently enriched in polyadenylated RNA (poly A+ RNA) using the MicroPoly(A) Purist Kit (Ambion). Following poly A+-enrichment, yield was quantified on a Nanodrop ND-1000 spectrophotometer. Double-stranded adapter-ligated cDNA was prepared from poly A+ RNA using the Marathon cDNA Amplification Kit (Clontech).

Cloning of Nuclear Receptors: sequence similarity between known vertebrate and cnidarian nuclear receptors was used to design degenerate oligonucleotide primers toward three groups of nuclear receptors: NR1, NR2/6, and NR7 (Table 1). Primers and nuclear receptors were named based on similarity to nuclear receptors in *A. millepora* (Grasso *et al.* 2001). Nuclear receptor cDNA fragments were amplified in two rounds of RT-PCR. In the first round, 25 µl reactions containing 1 ul cDNA, 25 µM primers, 4 mM $MgCl_2$, and 0.875 U AmpliTaq Gold Polymerase in GeneAmp PCR Buffer (Applied Biosystems) were subjected to the following cycling conditions in a Perkin-Elmer

TABLE 1

Degenerate oligonucleotide primers used in initial cloning of P. damicornis nuclear receptors. The following standard abbreviations are used: K = G or T, M = A or C, R = A or G, S = C or G, W = A or T, Y = T or C

Primer Name	Sequence	Use
dNR1_F415	AAR CAT TAC GGR ATY TAY GC	Cloning PdNR1
dNR1_R278	AA GCA YTT CYT CAR YCG RCA	Cloning PdNR1
dNR2_310F	GAR GGW TGC AAR RGM TTC TTC	Cloning PdNR6
dNR2_466R	CAG CTT CYT TSA GCA TKC C	Cloning PdNR6
dNR7_F226	CAR CAY CAC CGN AAY CAG TG	Cloning PdNR7
dNR7_R504	CTC GCA CAT GTT YTC RAT KCC	Cloning PdNR7

GeneAmp 2400 thermocycler: 94 °C/10 min, (94 °C/15 s, 62 ºC/30 s, 72 °C/30 s) for 40 cycles, 72 °C/7 min. An aliquot (1 µl) from the first round reaction was re-amplified in a 50 µl reaction for 30 cycles under identical cycling conditions. PCR products were cloned into pGEM-T Easy (Promega) and sequenced. DNA sequences were analyzed using the Wisconsin Package (GCG, Accelrys) and Bioedit Sequence Alignment Editor software (Hall 1999).

Gene-specific forward and reverse primers (Table 1) were designed based on the *P. damicornis* nuclear receptor partial sequences for use in 5'-/3'-RACE (rapid amplification of cDNA ends) reactions. Gene-specific primers, adapter primers, and adapter-ligated cDNA (produced with the Marathon cDNA Amplification Kit, Clontech) were used in PCR reactions according to the recommended touchdown PCR cycling conditions. Dimethylsulfoxide (DMSO) was added to the RACE reactions at a final concentration of 5%. RACE products were cloned and sequenced as described previously.

Phylogenetic Analysis: *P. damicornis* cDNA and deduced amino acid sequences were aligned with previously reported nuclear receptor sequences from *A. millepora* (Grasso *et al.* 2001, Accession numbers AF323680-AF323690) using Clustal X 1.81 with default parameters. The DNA binding domains of the aligned amino acids were used to create phylogenetic trees using parsimony and distance (minimum evolution) criteria using PAUP*4.0b10 software (Swofford 2003). A heuristic search strategy was used to create trees; branch swapping and tree-bisections reconnection were repeated to obtain bootstrapping values from 1000 replicates.

RESULTS

Using RT-PCR, partial cDNA and deduced amino acid sequences were determined for three nuclear receptor genes in *Pocillopora damicornis:* PdNR1, PdNR6, and PdNR7 (Fig. 2. GenBank Accession Numbers EF685356-8). Additional sequence information was obtained for PdNR6 by 3' RACE and for PdNR7 by 5'-RACE', so deduced cDNA and amino acid sequences were assembled from the overlapping sequences (Fig. 3). Each of the three nuclear receptors was detected in cDNA produced from *P. damicornis* collected both in Hawaii and in Costa Rica.

A 120-bp fragment of one *P. damicornis* nuclear receptor (PdNR1, GenBank Accession number EF685356) was cloned from total RNA using degenerate primers. PdNR1 is a predicted ortholog of a nuclear receptor cloned from *A. millepora* (AmNR1/Amtll). The fragment is highly similar (Table 2) to a portion of the AmNR1 DNA binding domain (DBD).

A 120-bp fragment of a second nuclear receptor was cloned and used in RACE reactions with gene-specific primers using adapter-ligated cDNA produced from poly A+-RNA. A 1318-bp product was amplified using

TABLE 2

Comparison of P. damicornis *nuclear receptors with putative orthologs from* A. millepora *(AmNR1, AmNR6, and AmNR7). Shown are the length of the amino acid alignment, the overall amino acid identity, and the percent amino acid identity by domain (A/B domain, DNA binding domain, hinge region, and ligand binding domain). The percent nucleotide identity within the DNA binding domain is indicated parenthetically. Within a domain, an asterisk indicates that only a partial sequence is available for* P. damicornis *and a dash indicates little or no sequence is available for* P. damicornis

	Alignment Length (AA)	Overall Identity	A/B	DBD (nt)	Hinge	LBD
PdNR1	39	97	--	97* (87)	--	--
PdNR6	304	86	--	100 (82)	85	83
PdNR7	168	93	75.9	99 (85)	94	--

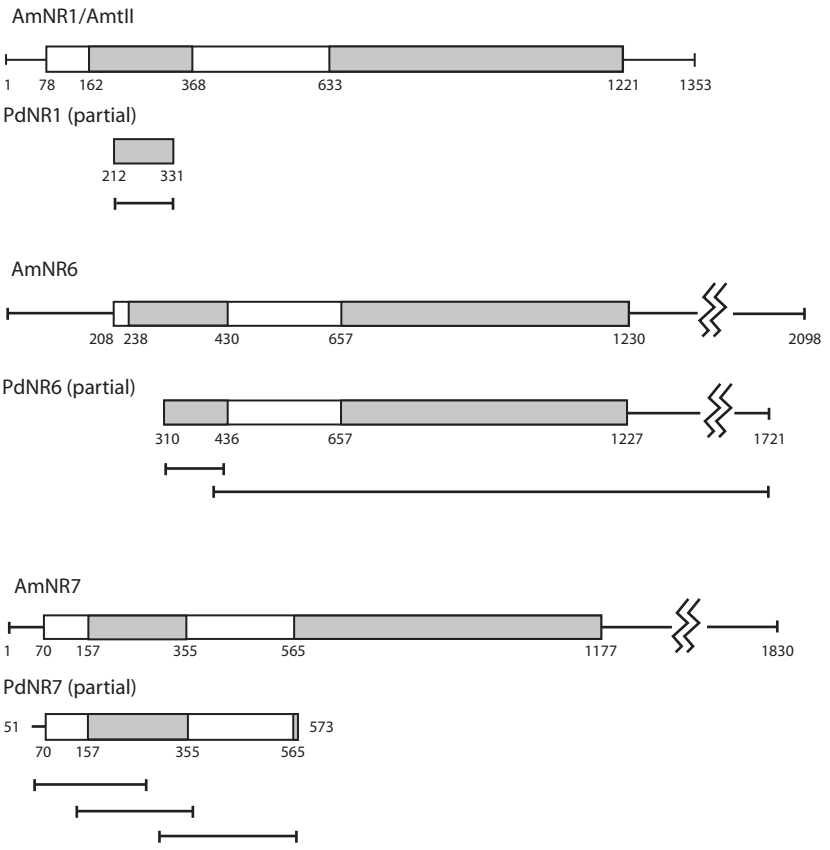

Fig. 2. Schematic representation of three nuclear receptors identified in *A. millepora* (Grasso *et al*. 2001) and *P. damicornis* (this study). For each pair of receptors, the *A. millepora* complete transcript is depicted at the top, with 5'- and 3'-UTR (untranslated region) indicated by solid lines and coding sequence indicated by a rectangle. *P. damicornis* and *A. millepora* sequences were aligned and numbered to indicate the number of nucleotides from the start of the *A. millepora* transcript. The DNA binding domain (DBD) and ligand binding domain (LBD) are shaded. Lines under the *P. damicornis* sequence indicate individual PCR products that were (PdNR6 and PdNR7) assembled.

3'-RACE. The predicted cDNA assembled from the two fragments (PdNR6, GenBank Accession number EF685357) was 1412-bp and represented the DBD (partial sequence), hinge region, ligand binding domain (LBD) and 3' untranslated region (3'UTR). The sequence shared 86 % overall amino acid identity with the aligned portion of AmNR6 in the coding region (Table 2).

A third nuclear receptor fragment (275 bp) was cloned using degenerate primers, and two overlapping fragments (227 bp, 226 bp) were amplified in successive 5'-RACE reactions. The predicted cDNA assembled from the three fragments (PdNR7, GenBank Accession number EF685358) was 523-bp and represented the 5'-UTR (may represent a partial sequence), A/B domain, DBD, hinge region and initial portion (2 residues) of the LBD. PdNR7 shares 93 % overall amino acid identity with the aligned portion of the AmNR7 coding region (Table 2).

The phylogenetic relationship of the *A. millepora* nuclear receptors to other vertebrate and invertebrate nuclear receptors has previously been examined in detail. In this

PdNR1

CDGCSGFFKRSIRRNRSYTCRATNGKGNCPVDKIHRNQY

PdNR6

FKRTVQKQLHYTCVENMSCQIDKNNRIRCQFCRFQKCLSLGMLKEAVRE
DRAPGGRPRIKSLIGMKENADTFVSSELITQLIQARPDATPKRRPDYLELGFTEI
CPLNPVEVVMELVLQEVDLILAWAFKVPGFRELNREDQASLVSTALLELLVLR
VCQRSSAHQGSVLLAKDVLLTPGHSFNLVLEQWSSQLACFAYKLQSLQLDMA
EFACVNAIMLFEQETSSGLKNREMVDFILNRSLDALRDYIKCSYPDKPSRFAHI
LLRLPTLRSVCSRMSNESLFAQSFLNMAVPQVLSFILETKT

PdNR7

MAVVPVSTWCTEKTEPVEEPSEKNVQQVE**CAVCGDKSSGKHYGVFTCEGC**
KSFFKRSVRRNLSYTCRASRNCPIDQHHRNQCQYCRLRKCIKVGMRREAV
QRGRIPPTQVPQPSPQHSALNGADVANGHSFLSGFISLLLRAEPYPTARFQQGL
NMPCGIMGIENICE

Fig. 3. Deduced partial amino acid sequences for three nuclear receptors in *P. damicornis*. The DNA-binding domain (DBD) is indicated in bold and the Ligand-binding domain (LBD) is underlined. The LBD is indicated beginning with α helix 3, a region that can be unambiguously identified, as in Grasso *et al.* (2001).

study, phylogenetic analysis was conducted primarily to support the predicted homologous relationships between pairs of receptors cloned from *A. millepora* and *P. damicornis*. Both parsimony (not shown) and minimum evolution (Fig. 4) criteria supported orthologous relationships of PdNR1 with AmNR1, PdNR6 with AmNR6, and PdNR7 with AmNR7.

DISCUSSION

In this study, PCR products corresponding to three distinct nuclear receptors were cloned from *P. damicornis* cDNA. These cDNA sequences are highly similar to three nuclear receptor sequences identified in the coral *A. millepora* (AmNR1, AmNR6 and AmNR7) (Grasso *et al.* 2001). The cDNA fragments isolated from *P. damicornis* are likely to be orthologs of these genes, and have been named accordingly (PdNR1, PdNR6, PdNR7). Two of the products amplified from *P. damicornis* (NR1 and NR7) are similar to known vertebrate nuclear receptors, as has been noted for *A. millepora* (Grasso *et al.* 2001). One (NR6) is more divergent and cannot

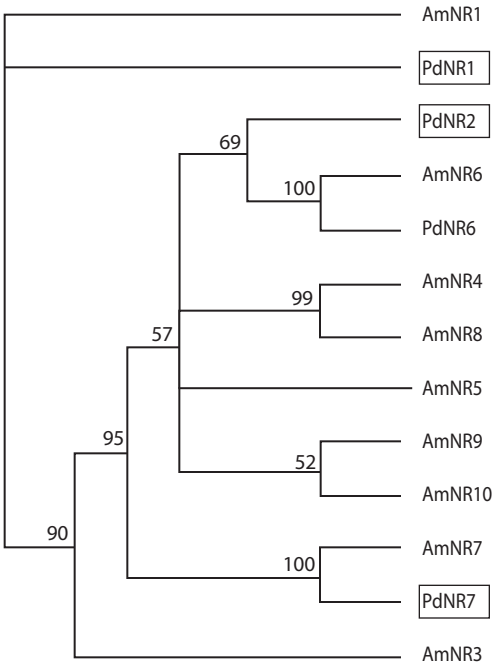

Fig. 4. Minimum evolution tree constructed using deduced amino acid sequences corresponding to the DNA binding domains of *A. millepora* and *P. damicornis* (boxed) nuclear receptors. Numbers indicate the percentage of 1000 bootstrap replicates that support the indicated topology.

be unambiguously associated with a known nuclear receptor subfamily.

PdNR1 and AmNR1 are putative homologs of the tailless genes in nuclear receptor subfamily 2E. Ligands have not been identified for tailless genes, which can be transcriptional activators or repressors (Steingrimsson *et al.* 1991, Yu *et al.* 2000). Tailless genes help to regulate nervous system differentiation in mammals and insects, and are particularly important for eye and forebrain development (Kobayashi *et al.* 1999, Yu *et al.* 2000, Roy *et al.* 2002, Stenman *et al.* 2003). In the mouse, some effects of TLX (a tailless gene) on vision and brain development are mediated through specific interactions with Pax genes (Pax2 and Pax6) (Yu *et al.* 2000, Stenman *et al.* 2003). Similar Pax genes have been described in cnidarians (Miller *et al.* 2000, Kozmik *et al.* 2003), so it is possible that some aspect of this interaction is conserved in coral. In addition, fax-1, a subfamily 2E nuclear receptor isolated from the nematode *C. elegans*, is essential for normal expression of FMRFamide-like neurotransmitters. A variety of FMRFamide-like neuropeptides have been characterized in cnidarians (Grimmelikhuijzen 1983, Grimmelikhuijzen & Spencer 1984, Grimmelikhuijzen *et al.* 1996, Moosler *et al.* 1997), but molecular regulation of their expression has not been described.

PdNR7 and AmNR7 are putative homologs of COUP-TF (chicken ovalbumin upstream promoter transcription factor, subfamily 2F). COUP-TFs have also been identified in the coral *Montipora capitata* (our unpublished data, Genbank Accession number AF254813), hydras, flatworms, sea urchin, and lancelets (Chan *et al.* 1992, Escriva *et al.* 1997, Langlois *et al.* 2000). COUP-TFs help to regulate development of muscles, the heart and the nervous system, particularly differentiation of the hindbrain and photoreceptors (Mlodzik *et al.* 1990, Fjose *et al.* 1993, Lu *et al.* 1994, Qiu *et al.* 1994). In most of these systems, COUP-TFs repress transcription of target genes. In hydra, COUP-TF is expressed in a subset of neural cells and acts as a negative transcriptional regulator in cell-based reporter assays (Gauchat *et al.* 2004, Miljkovic-Licina *et al.* 2004).

PdNR6 is homologous to AmNR6. These genes are related to another nuclear receptor in *A. millepora* (AmNR2). Grasso *et al.* (2001) found that AmNR2 and AmNR6 are related to two genes from the anemone *A. sulcata* (Escriva *et al.* 1997) but are not obvious homologs of any nuclear receptors described in bilaterian animals. These genes may represent a cnidarian-specific diversification and await further study. PdNR6, AmNR6 and AmNR2 lack a consensus AF2 domain and may be transcriptional repressors (Grasso *et al.* 2001).

None of the nuclear receptors identified in *P. damicornis* or *M. capitata* are apparent homologs of known ligand-activated nuclear receptors (i.e., receptors for steroids, retinoids, or other small molecules). A jellyfish nuclear receptor (jRXR) has been reported to bind 9-cis retinoic acid *in vitro*, but additional experiments are needed to determine whether other cnidarian nuclear receptors bind ligands. If binding of ligands regulates the activity of some cnidarian nuclear receptors, identifying these ligands would provide a means to assess whether exposure to environmental chemicals can disrupt cnidarian nuclear receptor signaling. On the other hand, if cnidarian nuclear receptors function without a requirement for ligands, it will be important to determine what other processes control their expression and transcriptional activity.

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RESUMEN

En el reino animal, los receptores nucleares modulan la transcripción para regular diversos procesos incluyendo el desarrollo embrionario, la homeostasis energética y la diferenciación sexual. Hasta la fecha, las secuencias de los receptores nucleares, se han registrado en sólo unas pocas especies cnidarian y poco se sabe acerca de los receptores nucleares en función de cnidarios. Hemos identificado tres genes de los receptores nucleares en colonias de coral de *Pocillopora damicornis* (PdNR1, PdNR6, y PdNR7). El cDNA y deducir las secuencias de aminoácidos indican que estos tres genes están estrechamente relacionados con los receptores nucleares se ha informado anteriormente en larvas plánula de *Acropora millepora* (AmNR1, AmNR6, y AmNR7). Ninguno de los tres receptores nucleares de *P. damicornis* son aparentes homólogos de receptores ligando-activados conocidos. Dos son los más estrechamente relacionados con los genes de vertebrados importantes en la diferenciación neuronal y la señalización (subfamilia 2E, los genes "sin cola" y subfamilia 2F, COUP-TFs). La tercera no puede ser inequívocamente clasificada sin una subfamilia reconocida en la actualidad. Son necesarios estudios futuros para describir las funciones de los receptores nucleares cnidarian en el desarrollo y la fisiología.

Palabras clave: receptores nucleares, *Pocillopora damicornis*, Costa Rica, corales.

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