



## RESEARCH ARTICLE

**REVISED** **The tropical coral *Pocillopora acuta* displays an unusual chromatin structure and shows histone H3 clipping plasticity upon bleaching [version 2; peer review: 2 approved]**

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**v2** **First published:** 29 Jul 2021, 6:195  
<https://doi.org/10.12688/wellcomeopenres.17058.1>  
**Latest published:** 25 Jan 2022, 6:195  
<https://doi.org/10.12688/wellcomeopenres.17058.2>

### Abstract





**Background:** *Pocillopora acuta* is a hermatypic coral with strong ecological importance. Anthropogenic disturbances and global warming are major threats that can induce coral bleaching, the disruption of the mutualistic symbiosis between the coral host and its endosymbiotic algae. Previous works have shown that somaclonal colonies display different levels of survival depending on the environmental conditions they previously faced. Epigenetic mechanisms are good candidates to explain this phenomenon. However, almost no work had been published on the *P. acuta* epigenome, especially on histone modifications. In this study, we aim at providing the first insight into chromatin structure of this species.

**Methods:** We aligned the amino acid sequence of *P. acuta* core histones with histone sequences from various phyla. We developed a centri-filtration on sucrose gradient to separate chromatin from the host and the symbiont. The presence of histone H3 protein and specific histone modifications were then detected by western blot performed on histone extraction done from bleached and healthy corals. Finally, micrococcal nuclease (MNase) digestions were undertaken to study nucleosomal organization.

**Results:** The centri-filtration enabled coral chromatin isolation with less than 2% of contamination by endosymbiont material. Histone sequences alignments with other species show that *P. acuta* displays on average ~90% of sequence similarities with mice and ~96% with other corals. H3 detection by western blot showed that H3 is clipped in healthy corals while it appeared to be intact in bleached corals. MNase treatment failed to provide the usual mononucleosomal digestion, a feature shared with some cnidarian, but not all; suggesting an unusual chromatin structure.


### Open Peer Review

**Approval Status**  

	1	2
<b>version 2</b> (revision) 25 Jan 2022		 <a href="#">view</a>
<b>version 1</b> 29 Jul 2021	 <a href="#">view</a>	  <a href="#">view</a>

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Any reports and responses or comments on the article can be found at the end of the article.

**Conclusions:** These results provide a first insight into the chromatin, nucleosome and histone structure of *P. acuta*. The unusual patterns highlighted in this study and partly shared with other cnidarian will need to be further studied to better understand its role in corals.

### Keywords

Pocillopora acuta, Pocillopora damicornis, invertebrate epigenetics, chromatin structure, Histone H3 clipping

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**Competing interests:** No competing interests were disclosed.

**Grant information:** This study is set within the framework of the Laboratoire d'Excellence (LABEX) TULIP (ANR-10-LABX-41) and was supported by a Wellcome Trust strategic award [107475]. This study was also supported by the French Agence Nationale de la Recherche through the Program BLOADAPT [ADACNI ANR-12-ADAP- 0016-03].

*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

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**How to cite this article:** Roquis D, Cosseau C, Brener Raffalli K *et al.* **The tropical coral *Pocillopora acuta* displays an unusual chromatin structure and shows histone H3 clipping plasticity upon bleaching [version 2; peer review: 2 approved]** Wellcome Open Research 2022, 6:195 <https://doi.org/10.12688/wellcomeopenres.17058.2>

**First published:** 29 Jul 2021, 6:195 <https://doi.org/10.12688/wellcomeopenres.17058.1>

**REVISED Amendments from Version 1**

Following one of the reviewer's comments regarding the possibility that the digestion pattern we observed in some species could originate from chromatin extraction issues, we have provided additional information and references showing that we tested a total of 24 different chromatin extraction techniques and obtained always similar results. Although one can never entirely exclude an experimental bias, this strengthens our interpretation that the most parsimonious explanation is that *P. acuta* has indeed a peculiar chromatin structure. We have also indicated future experiments which would allow to go beyond the results presented in this manuscript.

**Any further responses from the reviewers can be found at the end of the article**

## Introduction

Epigenetic modifications are good candidates to explain rapid, inheritable and reversible phenotypes without changes in the deoxyribonucleic acid (DNA) sequence (Danchin *et al.*, 2011). They range from chemical modifications of DNA (*e.g.* cytosine methylation), covalent changes of proteins participating to chromatin structure (*e.g.* histone modifications), as well as nuclear localization of chromosomes, and non-coding RNAs involved in post-transcriptional silencing of genes and repeated regions. These modifications have an impact on the chromatin structure, leading to the modulation of transcriptional activity (Wu & Morris, 2001). It is now clear that environmental factors can influence the epigenome to induce the expression of new phenotypes (Duncan *et al.*, 2014; Feil & Fraga, 2012).

In natural populations with genetic diversity, disentangling the respective roles of genetics and epigenetics in environmentally triggered phenotypes is hardly feasible, if not impossible, except with biological models with asexual reproduction. This rare feature is one of the characteristics of colonial organisms, such as corals, and they have been used as a model to address such questions. Most of these studies are focused on DNA methylation (Dixon *et al.*, 2018; Liew *et al.*, 2018) and non-coding ribonucleic acid (ncRNA) (Huang *et al.*, 2017; Huang *et al.*, 2019; Liew *et al.*, 2014), probably because the methods available to study these epigenetic marks are more straightforward. To date, only one article on Scleractinia corals has been performed at the histone modification level and has studied the broad nucleic content of the H2A.X and its phosphorylated form gamma-H2A.X (Rodríguez-Casariégo *et al.*, 2018). Many technical challenges make the epigenome analysis difficult: extraction of cells and tissues from the stony exoskeleton, working at a marine salt concentration to maintain cell, protein and chromatin integrity, and most importantly, separation of coral and Symbiodiniaceae biological material. Symbiodiniaceae are endocellular, and to focus on the coral epigenetic modifications, we can focus on non-symbiotic life stages, use naturally or artificially bleached colonies, or use laboratory techniques enabling physical separation of symbionts and host's nuclei and genetic material.

In this article, we describe a novel method to isolate coral nuclei (and by extension, coral chromatin) of *Pocillopora acuta*. We chose this coral for its worldwide distribution and ecological importance (Hoeksema *et al.*, 2015), its fast growth, allowing to quickly generate new colonies through propagations (Hughes *et al.*, 2015), and its documented response to thermal stresses (Vidal-Dupiol *et al.*, 2009; Vidal-Dupiol *et al.*, 2014). *P. acuta* has a genome size of approximately 325 Mb (Vidal-Dupiol *et al.*, 2019), while Symbiodiniaceae genome size is estimated at ~1,200–1,500 Mb (Lin *et al.*, 2015; Shoguchi *et al.*, 2013), which means that a single Symbiodiniaceae brings as much DNA as five coral cells, hence the necessity to reduce contamination from endosymbiotic material at the lowest possible level. We then used the isolated coral nuclei for the analysis of histone modifications and provide a first insight about the chromatin structure of this species. We also show that rupture of symbiosis (bleaching) is associated with changes in histone H3 structure (H3 clipping).

## Methods

### Ethics approval

The Direction Départementale de la Cohésion Sociale et de la Protection des Populations (DDSCPP) provided the permit N°C66-136-01 to IHPE for experiments on animals. Housing, breeding and animal care were done following the national ethical requirements (<https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32010L0063>).

### Biological material

The *Pocillopora acuta* isolate used in the present study was harvested in Lombok (Indonesia, CITES number: 06832/VI/SATS/LN/2001) and maintained at the Banyuls Aquarium (France) under optimal conditions (~26°C, pH 8.2, NO<sub>3</sub><0.1 mg/L, PO<sub>4</sub><0.01 mg/L, Ca<sup>2+</sup> ~450 mg/L, Mg<sup>2+</sup> ~1350 mg/L, KH ~7, PAR ~150 μmol photon/m<sup>2</sup>/s, 12h/12h light dark photoperiod, water motion of 30X tank volume per hour). Previously assigned to *Pocillopora damicornis*, this isolate was reassigned to *P. acuta* (Vidal-Dupiol *et al.*, 2019). Bleached coral colonies were obtained through a menthol treatment. Briefly, the colonies (5 colonies of ~5 cm in diameter) were placed in a four-liter tank filled with seawater. Water motion was created using a submerged water pump (set at ~100 L/h, EHEIM Compact ON 300), temperature was maintained at 27°C and light adjusted to 75 μmol/m<sup>2</sup>/s (PAR). The protocol for the menthol treatment was adapted from Wang *et al.* (2012). The first day, the corals were subjected to a menthol concentration of 0.58 mmol/L for 6h. After this exposure, they were transferred to the coral nursery for a 18h recovery period. During the second day, the same protocol was applied (menthol treatment and recovery step). The third day, the corals were exposed again to the same treatment, but only until the polyps were closed. Once the polyps were closed, the corals were placed in the coral nursery for recovery and Symbiodiniaceae loss. This last step typically takes four to five days. In this experiment, the coral colonies were left to bleach for five days. Once the polyps open up again, colonies were sampled. Healthy and bleached coral fragments were immediately frozen and stored in liquid nitrogen.

In this work, we also employed other *Cnidaria* for comparison in the chromatin extraction and digestion experiments. We chose three other hermatypic corals, *Stylophora pistillata* (CITES number IAZ3924), *Montipora digitata* (CITES number IEZ0069) and *Euphyllia divisa* (CITES number IUZ1609). We also used *Aiptasia* sp. and *Anemonia manjano*, two sea anemones (*Cnidaria* phylum), sharing an endosymbiotic relationship with Symbiodiniaceae, but without an aragonitic skeleton. All these species were acquired from the Canet en Roussillon aquarium (France). All of the species we used in this study are part of the Hexacorallia subclass. The anemones are from the Actinaria order and the corals from the Scleractinia order. Symbiodiniaceae cultures (*Cladocopium* sp. [type1] and *Fugacium* sp.) were obtained from the Roscoff Culture Collection (strain CCMP 2246, cat# RCC4017).

### *In silico* identification of canonical histones

We were interested in using isolated coral chromatin to perform chromatin immunoprecipitation (ChIP) on histone modifications. Before starting experiments, we wanted to confirm that histones were present in *P. acuta* genome, and that their amino acid sequences would be similar enough to other metazoan to be able to target them with commercial antibodies. Using Mega X (Kumar *et al.*, 2018) with the ClustalW algorithm under default parameters, we aligned the *P. acuta* predicted histone protein sequences (Vidal-Dupiol *et al.*, 2019) to reference protein sequences from *Mus musculus*, *Schistosoma mansoni*, a platyhelminth for which our laboratory has extensive experience in ChIP, and four other *Cnidaria*: *Pocillopora damicornis*, *Acropora digitifera*, *Nematostella vectensis* and *Hydra vulgaris*. Both *P. damicornis* and *A. digitifera* are from the same Scleractinia order as *P. acuta*. Accession numbers for each species are in Table 1.

### Western blots

Histones were either acid-purified with the Abcam histone extraction kit (cat #ab113476) from bleached and healthy coral fragments of approximately 5 mm of diameter (~ 0.5 g), or, for

rapid extraction, coral fragments were put into 1.5 mL tubes filled with 500  $\mu$ L of a solution containing 62.5 mM TRIS/Cl pH 6.8, 3% SDS, 10% sucrose, 0.2 M dithiothreitol (DTT) and 1.25 mM sodium butyrate. Tubes were sonicated using a Vibra Cell 75185 at 70% amplitude, 3 times 15 seconds, on ice. A cooldown on ice of 30 seconds was done between each sonication. *Dinoflagellata* phylum, which includes Symbiodiniaceae, possess core histones, but they have been documented to be larger and with different lateral chains than most metazoan (Marinov & Lynch, 2015). In previous experiments, we systematically failed to obtain a signal from western blots with commercial anti-histone antibodies on Symbiodiniaceae. Knowing this, we decided to use Symbiodiniaceae histone extraction as a negative control (to be certain the signal we observe in healthy and bleached *P. acuta* truly comes from the coral and not its symbiotes). We chose hamster (*Mesocricetus auratus*) as a positive control, as most antibodies are commercially developed and tested on mammals. Hamster brain and cultured aposymbiotic Symbiodiniaceae protein extracts were processed the same way as *P. acuta*. The laboratory has permission A 66040 from both French *Ministère de l'Agriculture et de la Pêche* and *French Ministère de l'Education Nationale de la Recherche et de la Technologie* for experiments on animals and certificate for animal experimentation (authorization 007083, decree 87-848 and 2012201-0008) for the experimenters. Housing, breeding and animal care follow the national ethical requirements.

The extract was cleared by centrifugation for 30 minutes at 1500 g, and the supernatant was collected. Total proteins (5 mg per sample) were mixed with Laemmli buffer (final concentration 1X) and denatured at 99°C for 5 mins. ECL™ Full-Range Rainbow (Amersham cat# RPN800E) was used as a molecular weight marker. Protein separation was done on 10% SDS-PAGE gel electrophoresis before being blotted on a nitrocellulose membrane (Trans-Blot turbo, Bio-Rad). The membrane was blocked with 5% non-fat dry milk in tris-buffered saline containing 0.05% tween 20 (TBST) one hour at room temperature.

**Table 1. Histone protein sequences accession numbers.** List of accession numbers of canonical histones amino acids sequences used for multiple alignment. Sequences for *Pocillopora acuta* come from the recently assembled genome (see Vidal-Dupiol *et al.*, 2019 for the complete data).

	H2A	H2B	H3	H4
<b><i>P. acuta</i></b>	TCONS_0002941	TCONS_00004949	TCONS_00016826	TCONS_00024865
<b><i>P. damicornis</i></b>	XP_027055899.1	XP_027035879.1	XP_027053709.1	XP_027055898.1
<b><i>A. digitifera</i></b>	XP_015747526.1	XP_015757821.1	XP_015766372	XP_015748868.1
<b><i>N. vectensis</i></b>	XP_001626711.1	XP_001617661.1	XP_001623766.1	XP_001630070.1.1
<b><i>H. vulgaris</i></b>	XP_002158325.1	XP_002156085.2	XP_002154470.1	XP_004205459.1
<b><i>S. mansoni</i></b>	XP_018651265.1	XP_018644054.1	XP_018646598.1	XP_018647790.1
<b><i>M. musculus</i></b>	AAA37763.1	NP_783594.1	NP_038576.1	NP_291074.1

One of the following primary antibodies, diluted in 5% non-fat milk in TBST was used for each western blot: anti-histone H3 (Abcam cat# ab1791 lot# 784471, polyclonal, dilution 1/500), anti-H3K4me3 (Millipore cat# 04-745 lot# 2326991, monoclonal, dilution 1/1000), anti-H3K9ac (Millipore cat# 07-352, lot# 2203126, polyclonal, dilution 1/500), anti-H3K27me3 (Diagenode, cat# C15410069, lot# A1821D, polyclonal, dilution 1/500), and anti-H3K27ac (Abcam cat# ab4729, lot# GR150-367-2, polyclonal, dilution 1/500), anti-H3K36me2 (Abcam cat# ab1220 lot# GR75522, monoclonal, dilution 1/500). After incubation, membrane was washed 3 times for 10 minutes in TBST. It was incubated with secondary antibody (peroxidase conjugated, goat purified anti-rabbit IgG [Pierce cat# 31460, lot# HB987318, polyclonal]) diluted 1/5000 in TBST for 1 hour. After washing 3 times for 10 minutes in TBST, the detection was carried out using the ECL reagents and the ChemiDoc MP Imaging system (BioRad). Estimation of the relation between number of amino acid residues and molecular weight in kD was done with [Calctool](#).

### Coral nuclei isolation

Our chromatin extraction protocol is based on [Cosseau & Grunau \(2011\)](#), but had to be adapted so that salinity, ionic strength and osmolarity of various buffers would match those of seawater (see [Table 2](#) for the exact composition of all buffers). *P. acuta* (healthy or bleached), *M. digitata*, *S. pistillata* and *E. divisa* polyps were removed from the aragonite skeleton using an Airpick. This instrument is custom-designed to gently blow air through a pipet tip. Advantage of the Airpick is that it can be used with small volume of extraction solution and with a low amount of biological material. Extractions with Airpick were performed on ice for 5 minutes (or until no tissues were left on the skeleton) with the coral fragments (approximately 5 mm of diameter and 0.5 g each) inside a 50 mL tubes

or small sealed plastic bags filled with 10 mL of a buffer 1 (see [Table 2](#) for composition of all the buffers used in this article). Tubes were then centrifuged at 800 g at 4°C for 10 minutes. Supernatant was carefully removed, and pellets were gently resuspended in 1 mL of buffer 1 and 1 mL of buffer 2. The mix was transferred in Dounce homogenizer and ground on ice for 4 minutes with pestle A, and then left to rest on ice for 7 minutes. Liquid was transferred on corex tubes already containing 8 mL of buffer 3, in a manner that the homogenate would form a layer on top of buffer 3. The two solutions have a different density, which allow them to stay one on top of the other without mixing. Corex tubes were centrifuged 20 mins at 4°C and 7,800 g, with the lowest break speed possible. This step allows separating *P. acuta* nuclei, which will form a pellet at the bottom of the tube, from cell debris and Symbiodiniaceae cells, which stay at the interphase between the two buffers. Supernatant was completely removed by pouring out of the tubes and then by aspiration with micropipette. Pellets, containing coral nuclei and chromatin, were used for chromatin digestion (with the objective of using digested chromatin for ChIP).

200 µg of *Aiptasia sp.* and *A. manjano* samples were directly ground in Dounce homogenizer with 1 mL of buffers 1 & 2, and then processed the same way as *P. acuta*.

### Chromatin digestion

Nuclei pellets (bleached and healthy *P. acuta*, *A. pallida* and *A. manjano*) from the coral nuclei isolation step were resuspended in 1.5 mL of MNase digestion buffer (see [Table 2](#) for composition).

Twenty microliters of the solution were kept for a coral nuclei and Symbiodiniaceae count (see step below). Aliquots of

**Table 2. Buffer composition.** Final concentration for all components of the various buffers used in the chromatin extraction and micrococcal nuclease (MNase) digestion.

	Buffer 1	Buffer 2	Buffer 3	MNase Buffer
KCl	30 mM	30 mM	30 mM	
NaCl	500 mM	500 mM	500 mM	500 mM
MgCl <sub>2</sub>	2.5 mM	2.5 mM	2.5 mM	40 mM
Tris/Cl pH 7.4	15 mM	15 mM	15 mM	10 mM
Sucrose	1 M	1 M	1.5 M	1 M
Sodium butyrate	5 mM	5 mM	5 mM	5 mM
Dithiotreitol (DTT)	25 mM	25 mM	25 mM	
Phenylmethanesulfonyl fluoride (PMSF)	0.5 mM	0.5 mM	0.5 mM	0.1 mM
cComplete protease inhibitor (Roche cat #11836145001)	1 X	1 X	1 X	
NP-40		0.3 %		
CaCl <sub>2</sub>				10 mM

250  $\mu$ L were prepared in Eppendorf tubes for digestion. One  $\mu$ L of 1 U/ $\mu$ L MNase (Affymetrix cat# 70196Y) was added to each tube, and incubation at 37°C was done for 0, 2, 4, 6 and 8 minutes. We also prepared a negative control with no MNase and incubated it at 37°C for 8 minutes. Because *Aiptasia* sp., *A. manjano*, *M. digitata*, *E. divisa* and *S. pistillata* were used with a comparison purpose, we only performed the digestion with three time points: 0, 4 and 8 minutes. Digestion was stopped with the addition of 20  $\mu$ L of 1M EDTA and tubes were left on ice for 5 minutes. The soluble fraction of chromatin was separated from cell debris by centrifugation at maximum speed (13,000 g) 10 minutes at 4°C, and DNA in the supernatant was purified using QIAquick PCR Purification kit (Qiagen cat# 28104) following the manufacturer's instructions. DNA fragments were eluted in 30  $\mu$ L of EB buffer (a Tris-EDTA buffer supplied with the kit). Twenty to thirty microliters of the purified DNA fragments were then separated by electrophoresis through a 1.8% agarose gel stained with ethidium bromide for 60 minutes at 100 V (in 0.5X Tris Borate EDTA buffer).

#### Estimation of *P. acuta* nuclei and intact Symbiodiniaceae in chromatin pellets

The chromatin extraction method we used was gentle enough to break *P. acuta* cytoplasmic membrane without lysing their nuclei or Symbiodiniaceae cells. Coral nuclei were then isolated from cell debris and Symbiodiniaceae due to the density difference of the solutions used during the centrifugation step in Corex tubes (step above). To get an estimation of the amount of possible Symbiodiniaceae contamination in the nuclei/chromatin pellet when using healthy *P. acuta* as starting material, we took 20  $\mu$ L of coral nuclei resuspended in MNase digestion buffer and added Hoechst 33342 (Invitrogen cat# C10329) at a final concentration of 1/2,000. We put 10  $\mu$ L on a Thoma cell counting chamber and observed on a fluorescent microscope (Leica DMLB) with objective PL Fluotar 40x and 100x at 350 nm. Hoechst 33342 stains coral nuclei with a blue fluorescence, but does not enter intact Symbiodiniaceae, which display a red fluorescence at 350 nm due to their chlorophyll content.

## Results

### Coral nuclei are isolated with less than 2% contamination of Symbiodiniaceae

Observation under fluorescent microscope showed that coral nuclei were not degraded and that there was little cell debris. We counted the number of intact Symbiodiniaceae (which have a red fluorescence at 350 nm because of their chlorophyll content) and intact coral nuclei (with blue fluorescence caused by Hoechst 33342 binding to DNA). We observed the result of 3 different nuclei isolation and never found more than 2 intact Symbiodiniaceae for every 98 coral nuclei (2%) (underlying data, supplementary file 3, Roquis *et al.*, 2021). The same result was observed in trials with *Aiptasia* sp. We observed, again through microscopy, that intact Symbiodiniaceae are stucked at the interphase between buffer 2 and buffer 3 during the centri-filtration, and do not pellet at the bottom of the tube with intact coral nuclei/chromatin. As expected, we did not find Symbiodiniaceae in menthol-bleached coral samples. The low amount of contamination with Symbiodiniaceae was confirmed by whole genome bisulfite sequencing (WGBS),

in another experiment from our group using the extraction technique presented in this article: less than 0.004% of the total reads aligned to the *S. minutum* and *S. kawagutii* genomes, which are known to compose the Symbiodiniaceae clades in our *P. acuta* population (Vidal-Dupiol *et al.*, 2019).

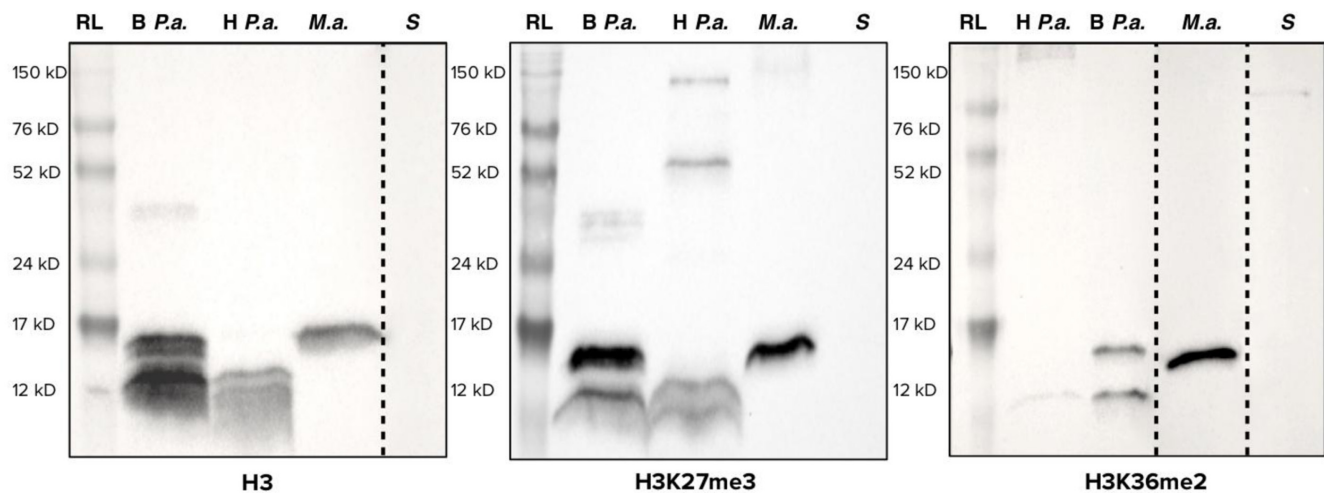
### *P. acuta* possesses histones, but displays an unusual chromatin structure that is also found in *E. divisa* and *A. manjano*

Protein sequences for the four core histones were found in *P. acuta* genome (Vidal-Dupiol *et al.*, 2019) and were aligned to other species to see similarity in amino acid sequences (underlying data, supplementary file 1, Roquis *et al.*, 2021). *P. acuta* H3 and H4 have 100% and 99% identity in their amino acid sequences with *M. musculus*. Other two core histones H2A and H2B have respectively 87% and 81% of identity with *M. musculus*. Histone tails for H3 were completely identical between *M. musculus* and *P. acuta*, while a single difference (valine at position 21 was substituted by an isoleucine) was observed in the histone tail of H4. This modification of histone H4 tail was also present in *P. damicornis*, *A. digitifera*, *H. vulgaris* and *N. vectensis*. Sequence similarities were high for the four histones when compared those of the blood fluke *S. mansoni*, and similarities increased when sequence were compared with more phylogenetically related organisms (*H. vulgaris* and *N. vectensis*). Histones H2A, H3 and H4 are completely identical between *P. acuta* and *P. damicornis* (a very similar species of the same genus), while the histone H2B amino acid sequence has 98% of identity between the two species. When compared to those of *A. digitifera*, another hermatypic scleractinian coral, 98% identity was found for *P. acuta* H2A protein, 87% with H2B, 97% with H3 and 100% with H4 (underlying data, supplementary file 1, Roquis *et al.*, 2021).

We then performed western blots to determine if commercial antibodies targeting histone modifications could be used on *P. acuta*. We tested hamster brain (positive control), crude protein extract of free Symbiodiniaceae, and bleached corals without Symbiodiniaceae. Symbiodiniaceae extract was used as a negative control, as their core histone sequences, and particularly their lateral chains, are fairly different from metazoan (Marinov & Lynch, 2015), and previous attempt by our group to detect them with commercial antibodies targeting histone modifications were always unsuccessful. Results of the western blots are shown on Figure 1.

Results for the three antibodies (anti-H3, anti-H3K27me3, anti-H3K36me2) were consistent between organisms. In all three cases, no signal was detected for Symbiodiniaceae, and a signal at the expected size (approximately 15 kDa) for histone H3 was observed in hamster. In healthy corals, a signal was observed, but at 12 kDa. Bleached coral displayed both the 12 kDa band and the expected 15 kDa band. The difference of about 3 kDa corresponds roughly to a difference of 25 amino acid residues in size, reminiscent of histone H3 clipping (Azad *et al.*, 2018; Santos-Rosa *et al.*, 2009).

To narrow down the clipping site, we used three more antibodies (anti-H3K4me3, anti-H3K9ac and anti-H3K27ac) on



**Figure 1. Western blots using antibodies targeting histone H3 (left), trimethylation of lysine 27 of histone H3 (H3K27me3, center) and dimethylation of lysine 36 of histone H3 (H3K36me2, right).** RL = Amersham ECL™ Full-Range Rainbow Marker; B *P. a.* = Bleached *Pocillopora acuta*; H *P. a.* = Healthy *Pocillopora acuta*; S = Symbiodiniaceae; M.a. = *Mesocricetus auratus*. For maximum readability, some lanes were cropped (shown with dashed lines), brightness and contrast were increased for H3K36me2. Original, uncropped and unedited, pictures of these western blots are available in the underlying data, supplementary file 2 (Roquis *et al.*, 2021).

acid-extracted purified histones and found the clipping to occur between amino-acid position 9 and 27 (Figure 2).

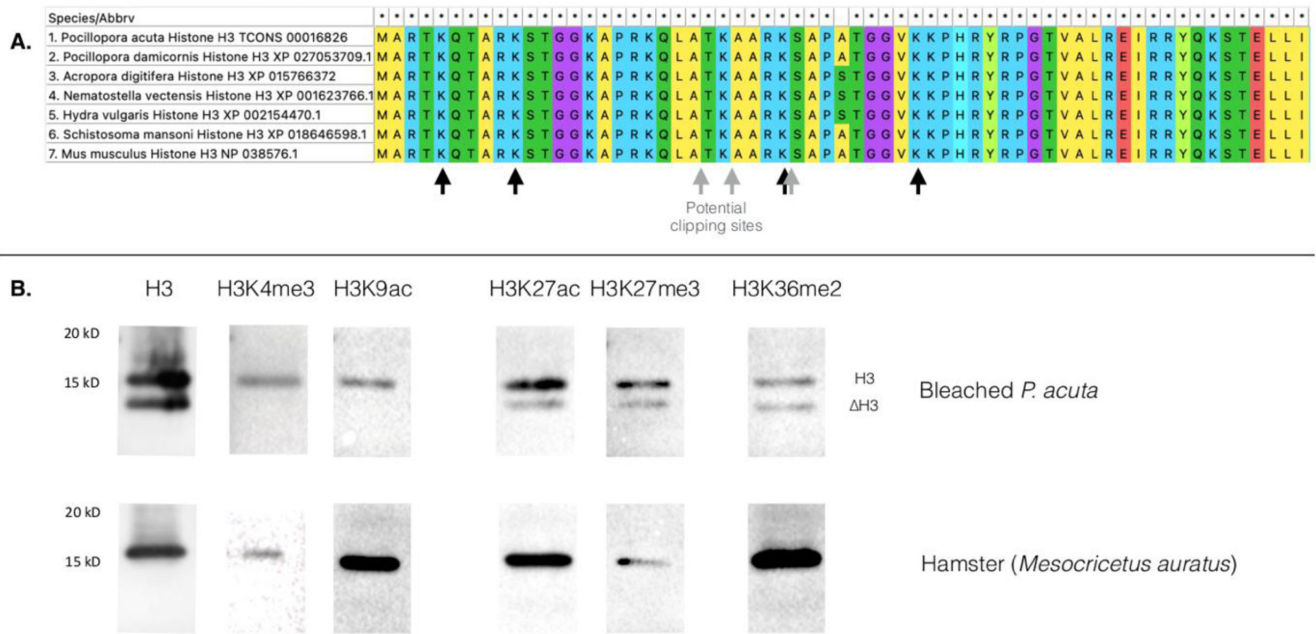
From these results, we suspected that there could be a different chromatin structure between bleached and healthy *P. acuta*, and we carried out a chromatin digestion with micrococcal nuclease (MNase). MNase digests DNA between nucleosomes, which creates DNA fragments with sizes that are multiples of approximately 150 bp (length of DNA wrapped around a nucleosome). When separated on a gel, the digested DNA looks like a ladder. After a prolonged digestion, only 150 bp fragments, corresponding to a mononucleosomal digestion would be detectable on an agarose gel. A very good example of this can be seen in Hewish & Burgoyne (1973). We did not observe this type of result for neither healthy nor bleached *P. acuta*. Instead, DNA is completely degraded with time, and no nucleosomal size fragments are seen (Figure 3).

To verify that it was not a problem with the chromatin extraction and digestion technique, we performed the same experiment on three other hermatypic corals *E. divisa*, *M. digitata* and *S. pistillata*, as well as two symbiotic anemones, *Aiptasia sp.* and *A. manjano*. As it can be seen on Figure 4, *E. divisa* and *A. manjano* present the same unusual digestion profile as *P. acuta*, while *S. pistillata*, *M. digitata* and *Aiptasia sp.* show the expected digestion profile, in the shape of a ladder and with an enrichment at 150 bp (corresponding to mononucleosomal fragments). The ladder profile is not striking in *A. pallida* (probably because of an excess of starting biological material), but enrichment around 150 bp is clearly visible.

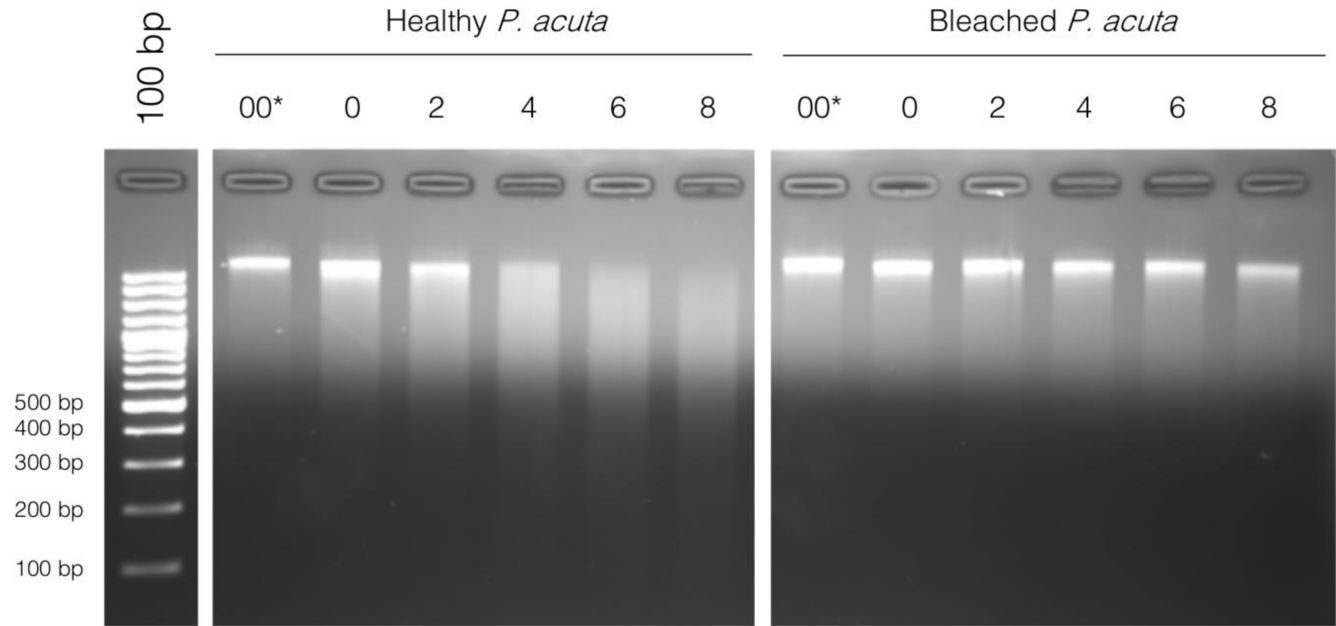
## Discussion

In this article, we describe (i) a straightforward method to separate *P. acuta* chromatin and DNA from Symbiodiniaceae

material, (ii) we provide the first description of *P. acuta* core histones, and (iii) we show that bleaching influences histone H3 structure in *P. acuta*. Alignments of protein sequences of histones H2A, H2B, H3 and H4, which compose nucleosomes, show that they are well conserved in *P. acuta*, with only little difference to mice. Interestingly, we did not observe the histone H3 band at the expected size (15 kDa) in healthy corals for any of the three antibodies we used (anti-H3, anti-H3K27me3, anti-H3K36me2). Instead, a single band of lower molecular weight (12 kDa  $\Delta$ H3) is visible. Bleached corals are different, with both the expected H3 band at 15 kDa, and the truncated one at 12 kDa. The size difference of the bands suggests clipping of H3 tails at roughly 25 residues. Clipping after alanine at position 21 (Ala21) in histone H3 was observed in *Saccharomyces cerevisiae*. Cleavage activity is induced under conditions of nutrient deprivation and sporulation (Santos-Rosa *et al.*, 2009). In the human parasite *Plasmodium falciparum*, H3 clipping occurs after Ala21 and is abundant in genes regulating DNA replication of the pathogen (Herrera-Solorio *et al.*, 2019). H3 cleavage was also observed in vertebrates where it is cell type-dependent and plays multiple important biological functions (Azad *et al.*, 2018). Two clipping sites are documented, one between lysine at position 21 (Lys-23) and alanine at position 24 (Ala-24) and the other between lysine at position 27 (Lys-27) and serine at position 28 (Ser-28) (Mandal *et al.*, 2013). Since we obtained a positive signal on Western blots for  $\Delta$ H3 with anti-H3K27me3, cleavage occurs downstream of position 27 in *P. acuta*. Mass spectrometry will be a next step to better identify the clipping site. Histone clipping is probably involved in the regulation of gene expression in a multitude of processes and is suspected to have a direct effect on chromatin structure (Azad *et al.*, 2018; Zhou *et al.*, 2014). One might argue that there is a differential protease activity in extracts of bleached and healthy colonies could be involved for our observations.

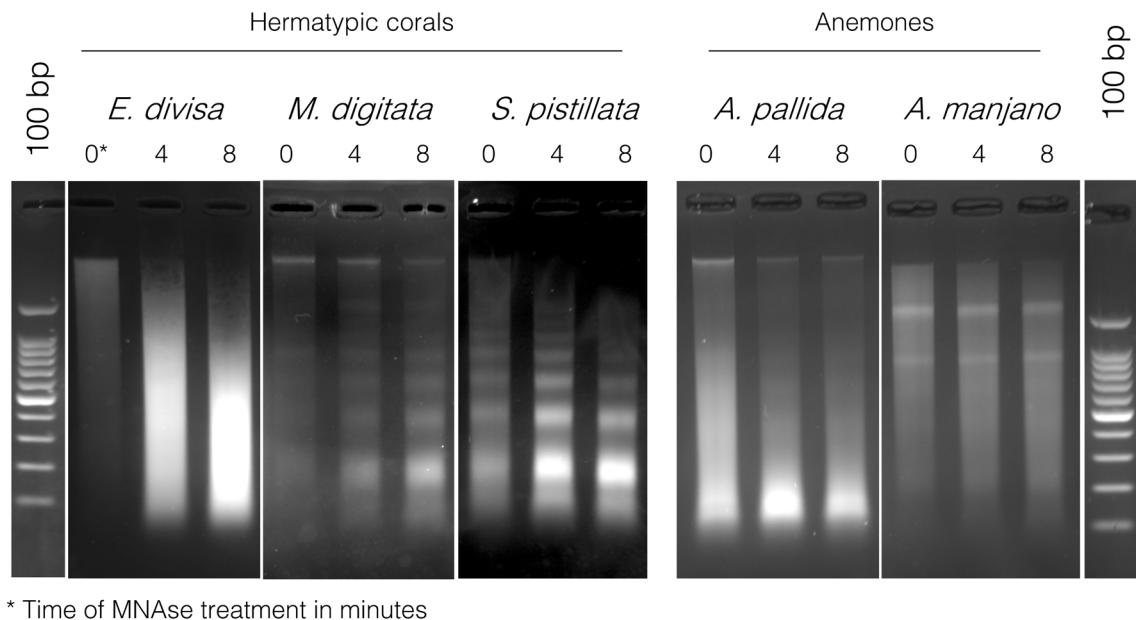


**Figure 2.** **A.** Partial amino acids alignment of histone H3 (see underlying data, supplementary file 1 for the complete alignment, Roquis *et al.*, 2021). Black arrows point at amino acid positions where chemical modifications are targeted by the antibodies we used (lysines at positions 4, 9, 27 and 36). Grey arrows potential histone H3 clipping sites based on literature data for other organisms. An asterisk (\*) means that the amino acid is conserved between all species **B.** Western blots using antibodies targeting histone H3, trimethylation of H3 lysine 4 and 27 (H3K4me3, H3K27me3), acetylation of H3 lysine 9 and lysine 27 (H3K9ac, H3K27ac), and dimethylation of lysine 36 of histone H3 (H3K36me2). Hamster *Mesocricetus auratus* is used as a positive control. For maximum readability, some lanes were cropped. Original, uncropped, pictures of these western blots are available in the underlying data, supplementary file 2 (Roquis *et al.*, 2021).



**Figure 3.** *Micrococcal* nuclease (MNase) digestion profile of chromatin from healthy and bleached *Pocillopora acuta* in function of digestion time (in minutes) at 37°C, ranging from 0 minutes to 8 minutes. 00 corresponds to chromatin left at 37°C for 8 minutes without the addition of MNase, and 0 is addition of MNase immediately followed by incubation on ice with addition of EDTA (enzyme inhibitor). 100 bp DNA ladder (Promega cat# G2120) is used for band size estimation. Uncropped gel pictures are available in the underlying data, supplementary file 2 (Roquis *et al.*, 2021).

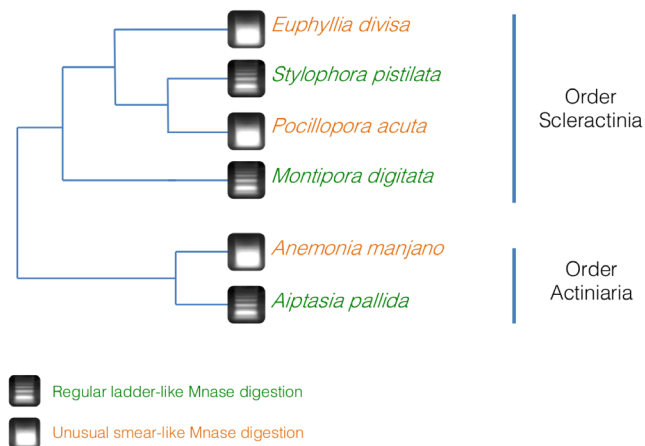




**Figure 4.** Micrococcal nuclease (MNase) digestion profiles for three hermatypic corals, *Euphyllia divisa*, *Montipora digitata* and *Stylophora pistillata*, as well as for the two sea anemones *Aiptasia sp.* and *Anemonia manjano*. 100 bp DNA ladder (Promega cat# G2120) is used for band size estimation. Uncropped gel pictures are available in the underlying data, supplementary file 2 (Roquis *et al.*, 2021).

We cannot formally exclude these possibilities, but the presence of highly efficient protease inhibitors should have prevented any proteolytic action during extraction. Surprisingly,  $\Delta H3$  is the major H3 isoform in healthy corals as opposed to bleached ones. We did not observe any signal in the Symbiodiniaceae extract, which confirms that the low molecular weight band (12 kDa  $\Delta H3$ ) we observe in healthy and bleached coral truly comes from *Pocillopora* and not from its Symbionts. It was thought for some time that Symbiodiniaceae did not possess real histones (Rizzo, 2003), but recent work demonstrated that they do have core histones, but usually of larger size and serving other functions than DNA packing and chromatin structure (Bayer *et al.*, 2012; Marinov & Lynch, 2015; Nand *et al.*, 2021; Roy & Morse, 2012). As we suspected, their protein structure is too different to be detected on Western blots by our commercial antibodies. Symbiodiniaceae live within coral cells, and their presence or absence may cause major cellular and nuclear reorganization as they induce significant transcriptome remodeling (DeSalvo *et al.*, 2010). There is the possibility that *P. acuta* chromatin may be compacted in different fashion, with other histone forms or histone-like proteins, depending on its symbiotic state. Recently, (Török *et al.*, 2016) have shown that *Hydractinia echinata*, another Cnidaria, possesses 14 different histones (different from the canonical H1.1, H2A.1, H2B.1, H3.1 and H4.1 histones), including a novel H3.3.2 variant. These histones variants are expressed at different life stages or in diverse tissues to efficiently pack DNA. It is plausible that a similar diversity exists in *P. acuta*, which should be investigated. Further analyses are needed to confirm these hypotheses and to investigate if this phenomenon is also present in the other corals and anemone species used in this study. Surprisingly, treatment of the

chromatin of *P. acuta* (healthy or bleached), *E. divisa* and *A. manjano* with MNase did not produce nucleosomal fragments. MNase cuts preferentially between nucleosomes because of steric hindrance. DNA is wrapped around nucleosomes for a 150 bp length, and incomplete digestion present a “ladder” profile on gels, with each band being a multiple of 150 bp, while complete digestion shows a unique, intense band at 150 bp (Hewish & Burgoyne, 1973; Hörz & Altenburger, 1981; Keene & Elgin, 1981; Noll & Kornberg, 1977; Wu *et al.*, 1979; Zaret, 2005). In these three species, DNA is completely digested by MNase (Figure 3 & Figure 4), similarly to what would happen in absence of nucleosomes (Hewish & Burgoyne, 1973). On the other hand, *Aiptasia sp.*, *M. digitata* and *S. pistillata*, gave the expected digestion profile for MNase. We cannot completely exclude that these differential digestion profiles might be caused by an effect of the chromatin extraction method that might be specific to *P. acuta*, *E. divisa* and *A. manjano*. However, we believe that this is unlikely because we tested a total of 24 different chromatin extraction methods which always provided consistent results of MNase digestions (see Roquis, 2015 at page 273 for the details of the chromatin extraction protocols). We conclude that some species within the Hexacorallia subclass have an unusual chromatin structure in which histones might be replaced by other proteins, or where nucleosomes have a different or weaker bond to DNA than what is commonly observed in other metazoan. However, these two digestion profiles are not coherent with the phylogeny of the species we used in this study (Daly *et al.*, 2003; Romano & Palumbi, 2009), implying that it is not a feature specific of an order or a genus (Figure 5). At the moment, we cannot state if this unusual chromatin structure is an ancestral feature of the Hexacorallia,



**Figure 5. Simplified phylogeny of the coral and anemone species used in this study, based on Daly *et al.* (2003) and Romano & Palumbi (2009) and associated with the results of the micrococcal nuclease (MNase) digestion.** The digestions profiles do not follow the phylogeny, hinting that the unusual chromatin structure profile is not an ancestral conserved trait in the Hexacorallia subclass.

which was lost in some species, or if it is a new character that appeared at multiple times within the Hexacorallia subclass. More analyses are needed in order to see if this unusual chromatin structure is limited to some species in the Scleractinia and Actiniaria orders, or if it is more widespread within Hexacorallia subclass or Anthozoa class.

## Conclusion

All together the results provided in this work represent a first inside into the chromatin, nucleosome and histone structure of *P. acuta*. We show here that the majority of H3 is clipped in healthy corals. Question remains about the function of this clipping, at which location it occurs, and the dynamics of the process. It will be necessary to conduct follow-up experiments such as immunofluorescence, ChIP and mass spectrometry to

answer these questions in the future. The unusual nucleosomal digestion patterns highlighted in this study and partly shared with other cnidarian will also need to be further studied in order to better understand its role and function in corals. This understanding will be necessary to address the essential question about the role of epigenetic modifications in rapid adaptation of these endangered organisms (Torda *et al.*, 2017).

## Data availability

### Underlying data

Zenodo: Suppl. Information to “The tropical coral *Pocillopora acuta* displays an unusual chromatin structure and shows histone H3 clipping plasticity upon bleaching”. <https://doi.org/10.5281/zenodo.5121858> (Roquis *et al.*, 2021).

This project contains the following underlying data:

- Supplementary File 1.pdf (Amino acids sequence alignments for the four core histones [H2A, H2B, H3, H4] in six species: *Pocillopora acuta*, *Acropora digitifera*, *Nematostella vectensis*, *Hydra vulgaris*, *Schistosoma mansoni* and *Mus musculus*.)
- Supplementary File 2.pdf (Original [uncropped and unedited] images used for Figures 1 to 4.)
- Supplementary File 3.xlsx (*P. acuta* nuclei and Symbiodiniaceae count on a Thoma cell counting chamber done over three different nuclei extractions.)

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

## Acknowledgments

The authors are very grateful for the permission to use the aquarium facilities of the UMS2348 for the sample generation. Earlier preprint versions of this manuscript can be accessed on bioRxiv (doi: <https://doi.org/10.1101/722322>) and TEL Archives Ouvertes (<https://tel.archives-ouvertes.fr/tel-01265805>).

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# Open Peer Review

Current Peer Review Status:  

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## Version 2

Reviewer Report 01 March 2022

<https://doi.org/10.21956/wellcomeopenres.19483.r48241>

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**Paola Furla** 

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**Eric Gilson**

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We acknowledge that the authors took into account our reserves in the interpretation of some the results in the discussion of their results. Therefore, we consider that the revised manuscript can be published.

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Coral symbiosis, biochemical signaling, genome stability

**We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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## Version 1

Reviewer Report 22 November 2021

<https://doi.org/10.21956/wellcomeopenres.18839.r46367>

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**Paola Furla** 

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This study focuses on the chromatin structure of the coral *P. acuta*. Overall, this study paves the way, both methodologically and conceptually, for future studies aimed at understanding coral chromatin plasticity facing environmental changes, a clear frontier in our knowledge of coral physiology.

As inferred from genomic data, the conservation of the four histones is very high in *P. acuta* (and other coral species), and almost identical to those of other animals, which is a known property of histone gene evolution.

The authors successfully used a method of host nucleus isolation (with less than 2% contamination with the symbionts).

By Western blotting, they show that the use of commercial antibodies, in particular those raised against post-translational modifications, are useable in coral studies. Interestingly, they observed that the full length H3 is seen only in bleached extract suggesting an H3 clipping processing in healthy colonies that is impaired upon bleaching.

By micrococcal digestion, no classical ladder of nucleosomes is visualized, suggesting a particular chromatin organization in this coral species.

Overall, the results are interesting, but the authors should be more prudent in their conclusions.

**Specific comments:**

- The authors could mention whether they detect histone gene clusters and histone variants in the coral genome and to what extent this echoes the situation in humans and mice.
- Figure 1: The authors should be more prudent regarding in the interpretation regarding clipping since the result could be explained by a differential protease activity during the extraction of bleached and healthy colonies
- Since the authors identified antibodies that differentially recognized the clipped and full length forms of H3, the authors could perform immunofluorescence experiments with these diagnostic antibodies on bleached and healthy coral nuclei to confirm that indeed the majority of H3 is clipped in healthy nuclei.
- The authors should more prudent regarding the interpretation of the negative results with micrococcal digestion. The fact that they were successful to produce a nucleosome ladder with other corals (e.g. *S. pistillata*) does not mean that, with their *P. acuta* nuclei, there are no extraction or digestion problems that prevent proper micrococcal nuclease digestion. A possible way to test that would be to mix *P. acuta* and *S. pistillata* nuclei prior to nuclease digestion and to analyze the ladder by differential hybridization on Southern blotting (assuming that differential probes exist between the two coral species). In any case, the authors should be more balanced in their conclusion.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Coral symbiosis, biochemical signaling, genome stability

**We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.**

Author Response 15 Jan 2022

**Christoph Grunau**, Univ. Montpellier, CNRS, Ifremer, Univ. Perpignan Via Domitia, Montpellier, France

**We are grateful towards the reviewer for taking the time to read our manuscript and providing the very constructive comments. We found that the comments were really pertinent and offer possibility of experiments we shall do in the future.**

- The authors could mention whether they detect histone gene clusters and histone variants in the coral genome and to what extent this echoes the situation in humans and mice.

**Initially, this was our goal at the beginning of our bioinformatics analyses on *P. acuta* genome. Unfortunately, the quality of the draft genome we have for *P. acuta* is low, and we observed that many loci with repetitive regions, including hypothetical histone variants, are often misassembled or truncated. We deemed not feasible with the current genome quality to properly list and compare known histone variants orthologs in *P. acuta*, and we chose to focus only on the canonical core nucleosomal histones as we had sufficient RNA-seq evidences to confirm we had the proper sequences for these four proteins. The *P. acuta* genome is still a work in progress, and we will definitely revisit this analysis once the overall quality will be improved.**

- Figure 1: The authors should be more prudent regarding in the interpretation regarding clipping since the result could be explained by a differential protease

activity during the extraction of bleached and healthy colonies

**We believe that the presence of protease inhibitors in the extraction buffers impede any protease activity. Both bleached and healthy corals were extracted several times, and the clipping phenomenon was constantly reproducible only in health corals. We have included this into the manuscript.**

- Since the authors identified antibodies that differentially recognized the clipped and full-length forms of H3, the authors could perform immunofluorescence experiments with these diagnostic antibodies on bleached and healthy coral nuclei to confirm that indeed the majority of H3 is clipped in healthy nuclei.

**The reviewer is right that many further experiments will be necessary to investigate in more detail the location and the function of histone clipping in corals. The experiments that were suggested are entirely justified and will answer some of the questions that remain to be answered in the future. Nevertheless, we believe to have clearly established that there is indeed differential clipping and we hope to have convinced the reviewer about at least this. We have added a comment about these open questions in the manuscript.**

- The authors should more prudent regarding the interpretation of the negative results with micrococcal digestion. The fact that they were successful to produce a nucleosome ladder with other corals (e.g. *S. pistillata*) does not mean that, with their *P. acuta* nuclei, there are no extraction or digestion problems that prevent proper micrococcal nuclease digestion. A possible way to test that would be to mix *P. acuta* and *S. pistillata* nuclei prior to nuclease digestion and to analyze the ladder by differential hybridization on Southern blotting (assuming that differential probes exist between the two coral species). In any case, the authors should be more balanced in their conclusion.

**We agree with the reviewer on this aspect. Our initial hypothesis when we observed the smeared MNase digestion pattern in *P. acuta* was that something went wrong with the chromatin extraction. We did not mention it in the manuscript, but we tried 24 different types of chromatin extractions on *P. acuta*, and always obtained the same digestion profile with the MNase (documented in <https://tel.archives-ouvertes.fr/tel-01265805> at page 273), which then convinced us that *P. acuta*'s chromatin was very likely to have a different structure. We included this information in the manuscript.**

**Competing Interests:** No competing interests were disclosed.

Reviewer Report 20 August 2021

<https://doi.org/10.21956/wellcomeopenres.18839.r45225>

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Maarten Dhaenens 

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In "The tropical coral *Pocillopora acuta* displays an unusual chromatin structure and shows histone H3 clipping plasticity upon bleaching", Roquis *et al.* describe their pioneering efforts in studying histones and to a wider extent the chromatin structure in (bleaching) coral. While still somewhat rudimentary in experimental design and techniques applied, the manuscript straightforwardly describes a novel and rather exciting finding on H3 clipping in coral.

Because of the limited amount of experiments presented, there is little to object to the way they were presented and interpreted. Indeed, a third wave of histone clipping manuscripts is currently being published (Dhaenens, M., *EMBO Reports*, 2021)<sup>1</sup> and especially histone H3 clipping is no longer a curiosity in the side line. While I do not want to promote self-citation, I do believe that our 2015 review on histone clipping and degradation really describes the importance of understanding this mechanism in as many organisms as possible.<sup>2</sup>

I have no specific comments on this publication, apart from being eager to seeing this unfold with more MS-based and possibly microscopy-based follow up experiments. If (the absence of) histone clipping turns out to play an important role in coral bleaching, trying to stimulate the protease activity (which is still not revealed for coral and could turn out to be yet another addition to the already long list of proteases) could even turn out to be beneficial and thus important for the future of marine sea life.

### References

1. Dhaenens M: Histone clipping: the punctuation in the histone code. *EMBO Rep.* 2021; **22** (8): e53440 [PubMed Abstract](#) | [Publisher Full Text](#)
2. Dhaenens M, Glibert P, Meert P, Vossaert L, et al.: Histone proteolysis: a proposal for categorization into 'clipping' and 'degradation'. *Bioessays.* 2015; **37** (1): 70-9 [PubMed Abstract](#) | [Publisher Full Text](#)

**Is the work clearly and accurately presented and does it cite the current literature?**

Partly

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Not applicable

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.



**Reviewer Expertise:** Histone research; histone clipping; mass spectrometry; data-independent acquisition technology

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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