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New Primers Reveal the Presence of a Duplicate Histone H3 in the Marine Turtle Leech Ozobranchus branchiatus

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New primers reveal the presence of a duplicate histone H3 in the marine turtle leech *Ozobranchus branchiatus*

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Introduction

Marine leeches, specific to sea turtles, have been implicated as potential vector organisms in the spread of fibropapillomatosis (FP), a pandemic neoplastic disease with green turtles (*Chelonia mydas*) having the highest affliction rate. Polymerase chain reaction identified two independent, seemingly functional histone H3 loci for marine turtle leeches *Ozobranchus branchiatus* collected from *C. mydas* in Florida and Hawaii. Primers were developed to amplify each product separately. These novel markers will be useful in identifying ectoparasites in FP research, evaluating other histone variants, and chromatin dynamics regulation studies.

Methods

- Methodology for sampling and morphological identification of *O. branchiatus* are given in McGowin *et al.* 2011.
- Nuclear protein coding-gene histone H3 were amplified using primers given in Rousset *et al.* (2007). When multiple products were visible in the chromatograms for the histone H3 gene, new reverse primers synthesized by Invitrogen Corporation (Carlsbad, CA, USA) were employed to obtain better sequencing results (Lavretsky *et al.* 2011):

  Reverse 1:H3R1 (5'-CCAACCAAGTACGCCTCA-3')
  Reverse 2:H3R2 (5'-CCAACCAAGTAAGCCTCG-3')

  H3R1 and H3R2 have an annealing temperature of 55ºC and functionally compatible with the H3af forward primer given in Rousset *et al*. 2007.
- Each 25-µL reaction mixture containing template, GoTaq® Green Master Mix, forward and reverse primers, and ddH_2O were prepared according to the GoTaq® Green Promega protocol as provided (http://www.promega.com).
- The PCR thermal regime for amplification was 94ºC for 7 minutes, followed by 45 cycles of 40 s at 94ºC, 40 s at a specific annealing temperature (52-55 ºC), 45 s at 72 °C, and then a final extension of 7 min at 72ºC using a PCR thermocycler (Eppendorf Mastercyclers).
- Purification of PCR Products was done using Agencourt® Ampure® XP Protocol 000387v001. However, after washing with 70% ethanol twice, PCR products were eluted using ddH_2O instead of elution buffer.
- Amplification products were sequenced in both directions using the Sanger Sequencing Method. Sequencing master mix included 4.5 µL ddH_2O, 1.75 µL Buffer (5X), 1 µL primer, and 1µL BigDye per reaction and ran at the following thermocycler settings: 96ºC for 1 minute, followed by 30 cycles of 10 s at 96ºC, 5 s at 50ºC, and 4 minutes at 60ºC, and then a final hold temperature at 4ºC. Buffer (5X) and BigDye provided by BigDye® Terminator v3.1 Cycle Sequencing Kit.
- Alignment analysis of genetic sequences was done using Sequencher™ 4.9 (Gene Codes, Inc.). A consensus maximum parsimony tree was generated using the Close-Neighbor-Interchange algorithm (Nei and Kumar 2000) in M EGA5 (Tamura *et al.* 2011).

References

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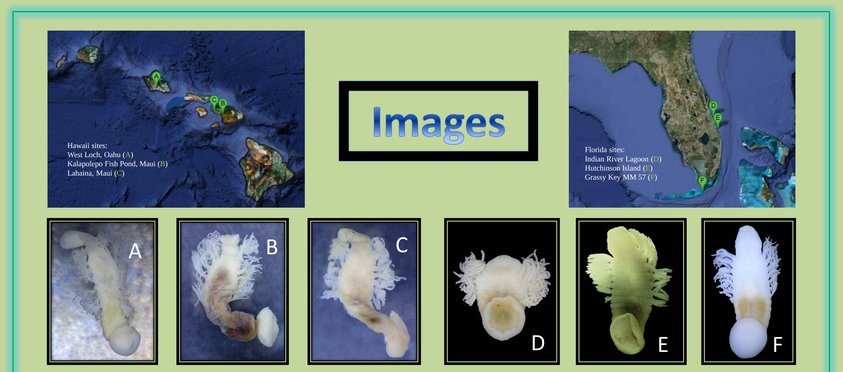
Table 1 Estimates of evolutionary divergence (Φ_{ST}) between histone H3 loci and *Ozobranchus branchiatus* populations

Histone H3 Haplotype	Hawaii R1	Florida R1	Hawaii R2
Hawaii R1	___	___	___
Florida R1	0.004	___	___
Hawaii R2	0.161	0.172	___
Florida R2	0.171	0.182	0.005

Conducted in MEGA5 (Tamura *et al.* 2011) using the Tamura 3-parameter model (Tamura 1992) with a gamma distribution (shape parameter = 5) and a total of 12 nucleotide sequences (259 base pairs/ sequence). Ambiguous positions were removed for each sequence pair.



Images of *O. branchiatus* from each sampling site are displayed above. Except for the *O. branchiatus* associated with the Indian River Lagoon location (specimen was collected on the same *C. mydas* as those sequenced), all photographed leeches were sequenced in this study.

Table 2
Histone H3 sequence accession data for specimens analyzed.

Higher taxa	Species	GenBank accession no.
ANNELIDA		
Clitellata		
Haplotaxida	*Allolobophora chlorotica*	FJ214261†, FJ374780†
	Antarctodrilus proboscidea	DQ779716‡
	Aporrectodea caliginosa	FJ214248†, FJ374784†
	Aporrectodea icterica	FJ214249†, FJ374781†
	Aporrectodea longa	FJ214250†, FJ214251†
	Aporrectodea rosea	FJ214252†, FJ214262†
	Aporrectodea tuberculata	FJ214268†, FJ374783†
	Dendrobaena attemsi	FJ214254†
	Dendrobaena clujensis	FJ214253†
	Dendrobaena hortensis	FJ214259†
	Dendrobaena veneta	FJ214264†
	Dendrodrilus rubidus	FJ214239†, FJ374782†
	Eisenia andrei	DQ779728‡
	Eisenia fetida	FJ214246†, FJ214247†, FJ214258†
	Eisenia lucens	FJ214255†
	Eisenoides carolinensis	FJ214256†
	Helodrilus oculatus	FJ214245†, FJ214271†
	Heronidrilus gravidus	DQ779736‡
	Lumbricus castaneus	FJ214244†, FJ214272†
	Lumbricus festivus	FJ214243†, FJ214270†
	Lumbricus rubellus	FJ214242†, FJ214257†
	Lumbricus terrestris	AF185262§, FJ214240†, FJ214241†, FJ214260†
	Octolasion lacteum	FJ214267†
	Octolasion tyrtaeum	FJ214263†, FJ214265†, FJ214266†, FJ214269†
Arhynchobdellida	*Erpobdella octoculata*	DQ779729‡
	Hirudo medicinalis	DQ779738‡
Rhynchobdellida	*Glossiphonia complanata*	DQ779733‡
	Helobdella stagnalis	DQ779735‡
	*Ozobranchus branchiatus**	JQ070406£123, JQ070407£45, JQ070408£6, JQ070409¥1, JQ070410¥23, JQ070411¥4, JQ070412¥8, JQ070413¥6

†Lund et al. 2008; §Rouse et. al. 1999; ‡Rousset et al. 2007; *This study: histone H3R1£; histone H3R2¥
Florida sites: Grassy Key MM 57†; Indian River Lagoon§; Hutchinson Island‡
Hawaii sites: Lahaina, Maui*; Kalapolepo Fish Pond, Maui¥; West Loch, Oahu£

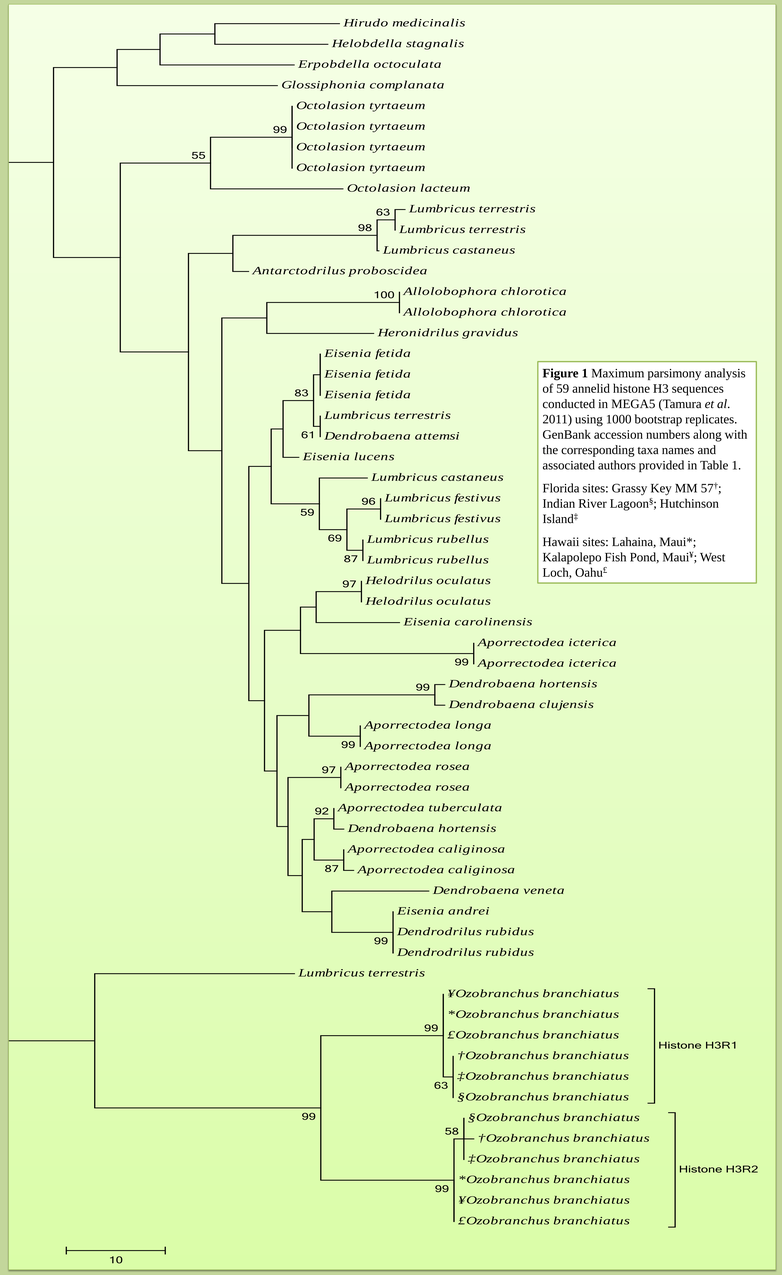


Figure 1 Maximum parsimony analysis of 59 annelid histone H3 sequences conducted in MEGA5 (Tamura *et al.* 2011) using 1000 bootstrap replicates. GenBank accession numbers along with the corresponding taxa names and associated authors provided in Table 1.

Florida sites: Grassy Key MM 57†; Indian River Lagoon§; Hutchinson Island‡

Hawaii sites: Lahaina, Maui*; Kalapolepo Fish Pond, Maui¥; West Loch, Oahu£

Results and Conclusions

- It is possible not all loci composing the histone H3 complex were identified and that pooling individuals resulted in false-positives for heterozygotes, but sequence divergence estimates (Φ_{ST}) ranging from 0.161 to 0.182 (Table 1) suggested at least two divergent loci (histone H3R1 and H3R2).
- The two well supported (≥ 99% bootstrap support) monophyletic groups (exclusively including either H3R1/H3R2 haplotypes) confirms the new locus-specific primers amplified two divergent loci (Figure 1). The two loci are sister to one another (99% bootstrap support) rather than to other annelids, suggesting the duplication event occurred within the Ozobranchidae lineage.
- Despite being phylogenetically distinctive, genetic diversity based upon location showed low levels of differentiation between Hawaiian and Floridian samples, with Φ_{ST} estimates of 0.004 and 0.005 for histone H3R1 and H3R2, respectively (Table 1). Indeed, there were two fixed differences between alleles sampled from Florida and Hawaii at H3R1 (Figure 1).

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http://www.chm.wright.edu/mcgowin/research/index.html