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Introduction

Fibropapillomatoeis (FP) is a panzootic plaguing all species of sea turtles with green turtles having the highest percentage of affliction.[1] Although fibropapilloma-associated turtle herpesvirus (FPTHV) has been identified as the causative agent of FP, the primary vector triggering this chronic tumor-forming disease is still unknown.[2] Parasitic marine leeches (*Ozobranchus* spp.) could be a potential mechanical vector behind the emergence of this panzootic, although it is uncertain if it is species specific. *Ozobranchus* spp. are very small and difficult to distinguish anatomically, and until now, there have been few attempts to document them. DNA barcoding using mitochondrial cytochrome *c* oxidase I (COI) gene as a molecular marker is a tool for identifying organisms and is especially useful for sea turtle leeches.[3] Two species of leeches present along the Florida Atlantic coast are *Ozobranchus branchiatus* and *Ozobranchus margoi*. The known hosts reported for *O. margoi* include a variety of sea turtle species, such as green (*Chelonia mydas*) and loggerhead (*Caretta caretta*) sea turtles, but the only reported host for *O. branchiatus* has been *C. mydas* (Refer to Figure 1).[4]

DNA barcoding was applied to samples of both species of leeches obtained from sea turtles captured at Indian River Lagoon and the St. Lucie Nuclear Power Plant in Florida (refer to Figure 1). DNA sequences were translated to protein sequences for alignment purposes. *O. margoi* is already present in the National Center for Biotechnology Information (NCBI) GenBank (AF003268), but leeches morphologically identified as *O. branchiatus* did not have a match in the genetic database. As a result, we used our DNA sequencing to add the *O. branchiatus* leech as a new species to GenBank (GU985465 and GU985466). There was a small but significant variance between the nucleic and amino acid sequence of the *O. branchiatus* leeches unexpectedly identified on a loggerhead and those found on green sea turtles, respectively from St. Lucie and Indian River Lagoon. The fact the *O. branchiatus* leech has changed specificity serves as an indication as to why the panzootic affecting mainly *C. mydas* initially has now appeared to a lesser degree in other turtle species.

Methods

Morphological Identification

- The following anatomical aspects were measured: number of gills on both sides, diameter of posterior sucker, and total length. Refer to Table 1.
- The leeches were morphologically identified based upon the number of gills; *O. branchiatus* has 7 gills, while *O. margoi* has only 5. Refer to Figure 1.

DNA Extraction and Purification

- Leeches were preserved in pure denatured ethanol. Prior to DNA extraction, all tissues were washed with double deionized water. Only tissues obtained from the gills, posterior, and anterior sucker of the leech were analyzed (about 13-25 mg) to avoid turtle blood contamination within the leeches' gut.
- DNA extraction and purification was performed using the procedures and materials provided in a Qiagen DNeasy Blood and Tissue kit (Qiagen, Inc.). Materials not provided in the kit included proteinase K (Qiagen, Inc.), RNase (Qiagen, Inc.), and ethanol.
- Purified genomic DNA was eluted by adding 200 µL Buffer AE to the same spin column in a new collection tube and centrifuged at 8,000 rpm for 1 minute. Centrifuge step was repeated once again using the same spin column and collection tube for a total of 400 µL volume.
- DNA samples were stored at -20 C and concentration measurement obtained using a ND-1000 NanoDrop Spectrophotometer (Thermo Scientific).

Polymerase Chain Reaction (PCR)

- COI gene was amplified using the following Universal Folmer primers (Invitrogen) originally 10µM in water:[5]

  LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') as forward primer

  HCO2198 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') as reverse primer
- Each primer was diluted to obtain a 50 µL working solution of 2.0 µM. A 50 µL working solution of AmpliTaq Gold 360 Master Mix (Applied Biosystems) was also prepared. All solutions were prepared in a biological safety cabinet (Labconco).
- 4 different leech samples were analyzed, each in duplicates:
  1) *O. branchiatus*, Indian River Lagoon, pooled DNA sample 1
     a) OBIRLAF1 (replicate 1)
     b) OBIRLAF2 (replicate 2)
  2) *O. branchiatus*, Indian River Lagoon, pooled DNA sample 2
     a) OBIRLGL1 (replicate 1)
     b) OBIRLGL2 (replicate 2)
  3) *O. branchiatus*, St. Lucie Power Plant, pooled DNA sample 1
     a) OBPP1 (replicate 1)
     b) OBPP2 (replicate 2)
  4) *O. margoi*, St. Lucie Power Plant, pooled DNA sample 1
     a) OMPP1 (replicate 1)
     b) OMPP2 (replicate 2)
- A total of 10 reaction tubes were set up as illustrated in Table 2. A second set of 10 tubes were prepared containing PCR reagents and labeled 1A-10A correspondingly.
- Contents of each "A" tube were mixed with the corresponding reaction tube of the same number for a total volume of 50 µL and placed in a thermocycler (Applied Biosystems). Refer to Table 3 for thermal cycling conditions.

Purification of PCR Products

- Purification of PCR products was performed using the procedures and materials provided in a QIAquick PCR Purification Kit.

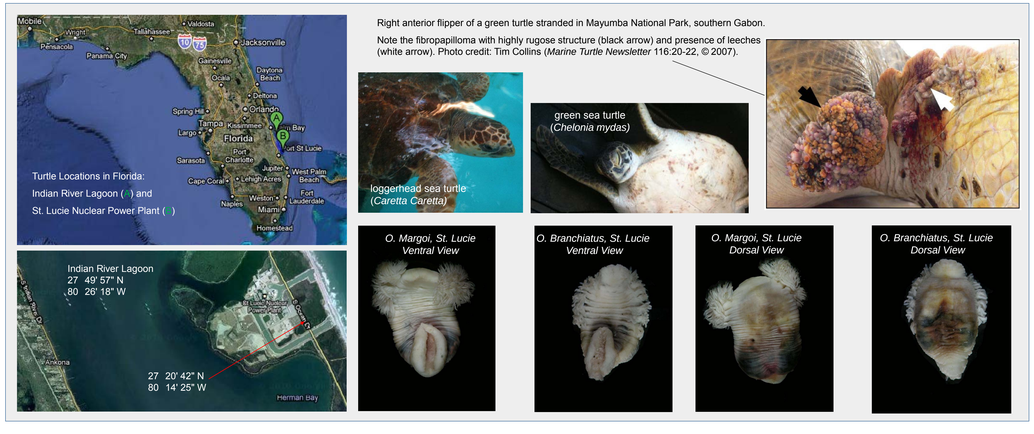
DNA Sequencing of PCR Products, Translation, and Alignment

- Double stranded DNA sequencing of PCR products was done by Retrogen, Inc. only on the first replicates and translated to protein sequences using EMBOSS Transeq. The invertebrate mitochondrial genetic code was used for translating DNA sequences.
- Alignment analysis of nucleic and amino acid sequences was done using Clustal W2. Based upon our chromatograms (refer to Figure 2) and alignment analysis for both forward and reverse genetic sequences, consensus DNA sequences were determined for *O. branchiatus* from Indian River Lagoon (OBIRL_consensus) and *O. branchiatus* and *O. margoi* from the St. Lucie Nuclear Power Plant (OBPP1 and OMPP1, respectively). Refer to Figure 3.
- DNA sequences were submitted as a nucleotide query through BLAST to determine if a match existed in the NCBI GenBank.

References

[1] Herbst L.H. Fibropapillomatiisis of marine turtles, Annual Reviews of Fish Diseases 4:389-425, 1994.
[2] Herbst L., Ene A., Su M., Desalle R., Lenz J. Tumor outbreaks in marine turtles are not due to recent herpesvirus mutations. Current Biology, 14(17):R697-R699, 2004.
[3] Hebert P.D.N., Cywinska A., Ball S.L., deWaard J.R. Biological identifications through DNA barcodes, Proc. R. Soc. Lond. B Biol. Sci.; 2003 Vol. 270, p313-322.
[4] Sawyer R.T., Lawler A.R., Overstreet R.M. Marine Leeches of the eastern United States and the Gulf of Mexico with a key to the species, Journal of Natural History 9:633-667, 1975.
[5] Folmer O., Black M., Hoeh W., Lutz R., Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates, Molecular Marine Biology and Biotechnology 3(5):294-299, 1994.

Figure 1



Right anterior flipper of a green turtle stranded in Mayumba National Park, southern Gabon.

Note the fibropapilloma with highly rugose structure (black arrow) and presence of leeches (white arrow). Photo credit: Tim Collins (*Marine Turtle Newsletter* 116:20-22, © 2007).

Table 1

Character (dimension in mm)	Specimens from Indian River Lagoon (documented FP on all hosts, *C. mydas*)				Specimens from St. Lucie Nuclear Power Plant (no documented FP on hosts, *C. caretta*)			
	December *O. Branchiatus* (12 leeches)		August *O. Branchiatus* (2 leeches)		August *O. Margoi* (2 leeches)	
	Range	Mean	Range	Mean	Range	Mean
Total Length	4.36-6.49	5.28 2.11	6	6	5-8	7 2
Diameter of posterior sucker	1.73-2.55	2.11 0.22	2	2	3	3

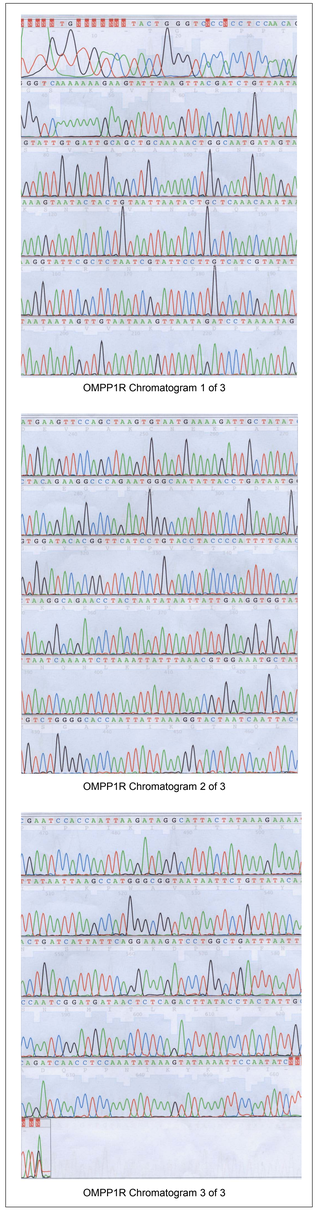
Table 3

PCR System 2700 Thermal Cycling Conditions			
Stage	Step	Temperature	Time
Holding	Denaturation	95 C	10 min
Cycling (35 cycles)	Denature	95 C	30 s
	Anneal	50 C	30 s
	Extend	72 C	60 s
Holding	Final extension	72 C	7 min
Holding	Final Hold	4 C	∞

Table 2

Sample and Blank Mixtures Preparation										
Reaction tube	Sample	DNA Conc. (ng/µL)	µL DNA template for ~ 100 ng	ng DNA template	µL Primer 1	µL Primer 2	Prep Tube	µL Master Mix	µL ddH2O	µL Total Volume
1	OBIRLAF1	27.2	3	81.6	5	5	1A	25	12	50
2	OBIRLAF2	27.2	3	81.6	5	5	2A	25	12	50
3	OBIRLGL1	29.1	3	87.3	5	5	3A	25	12	50
4	OBIRLGL2	29.1	3	87.3	5	5	4A	25	12	50
5	OBPP1	6.1	15	91.5	5	5	5A	25	0	50
6	OBPP2	6.1	15	91.5	5	5	6A	25	0	50
7	OMPP1	23.6	3	70.8	5	5	7A	25	12	50
8	OMPP2	23.6	3	70.8	5	5	8A	25	12	50
9	Primer blank	23.6	3	70.8	0	0	9A	25	22	50
10	DNA blank	0	0	0	5	5	10A	25	15	50

Figure 2



OMPP1R Chromatogram 1 of 3

OMPP1R Chromatogram 2 of 3

OMPP1R Chromatogram 3 of 3

Figure 3

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SeqA Name              Len(nt)   SeqB Name              Len(nt)   Score
=============================================================================
1    Genbank_margoi    651       2    OMPP1             684       99
1    Genbank_margoi    651       3    OBPP1             658       84
1    Genbank_margoi    651       4    OBIRL_consensus   658       84
2    OMPP1             684       3    OBPP1             658       84
2    OMPP1             684       4    OBIRL_consensus   658       84
3    OBPP1             658       4    OBIRL_consensus   658       96
=============================================================================

OBPP1          AATTTTATATTTTATATTTGGTGCCTGATCTGCAATAGTAGGCATAAGATTAAGAGTAAT 60
OBIRL_consensus AATTTTATATTTTATATTTGGTGCCTGATCTGCAATAGTAGGCATAAGATTAAGAGTAAT 60
Genbank_margoi -AATTTATACTTTATATTTGGAGGTTGATCTGCAATAGTAGGTATAAGTCTGAGAGTTAT 59
OMPP1          AATTTTATACTTTATATTTGAGGTTGATCTGCAATAGTAGGTATAAGTCTGAGAGTTAT 60

OBPP1          TATTCGATTAGAATTAAACCAACCAGGATCATTTTTAAATAACGATCAGCTGTATAATAC 120
OBIRL_consensus TATTCGATTAGAATTAAACCAACCAGGATCATTTTTAAATAATGATCAGCTGTATAATAC 120
Genbank_margoi CATCCGATTGGAATTAAATCAGCCAGGATCTTTCCTGAAATAATGATCAGTTGTATAACAG 119
OMPP1          CATCCGATTGGAATTAAATCAGCCAGGATCTTTCCTGAAATAATGATCAGTTGTATAACAG 120

OBPP1          TGTAATTACAGCACACGGTTTAATTATAATTTTTTTTATAGTGATACCAATCTTAATTGG 180
OBIRL_consensus TGTAATTACAGCACACGGTCTAATTATAATTTTTTTTATAGTAATACCAATCTTAATTGG 180
Genbank_margoi AATTATTACCGCCCATGGCTTAATTATAATTTCTTTATAGTAATGCCTATCTTAATTGG 179
OMPP1          AATTATTACCGCCCATGGCTTAATTATAATTTCTTTATAGTAATGCCTATCTTAATTGG 180

OBPP1          AGGTTTCGGTAATTGATTAGTACCTTTAATAATTGGTGCACCAGATATAGCATTTCCACG 240
OBIRL_consensus AGGTTTCGGTAATTGATTGGTGCCTTTAATAATTGGTGCACCAGATATAGCATTTCCACG 240
Genbank_margoi TGGATTCGGTAATTGATTAGTACCTTTAATAATTGGTGCCCCAGACATAGCATTTCCACG 239
OMPP1          TGGATTCGGTAATTGATTAGTACCTTTAATAATTGGTGCCCCAGACATAGCATTTCCACG 240

OBPP1          GTTAAATAACCTTAGATTTTGACTAATACCACCTTCTATAATTATACTAATTGGATCAGC 300
OBIRL_consensus GTTAAATAACCTTAGATTTTGACTAATACCACCTTCTATAATTATACTAATCGGATCAGC 300
Genbank_margoi TTTAAATAATTTAAGATTTTGATTAATACCACCTTCAATAATTATATTAGTAGGTTCTGC 299
OMPP1          TTTAAATAATTTAAGATTTTGATTAATACCACCTTCAATAATTATATTAGTAGGTTCTGC 300

OBPP1          ATTAATTGAAAACGGTGTCGGTACTGGTTGAACTGTTTACCCCCCCATTATCCTGGAAATAT 360
OBIRL_consensus ATTAGTTGAAAACGGTGTTGGAACTGGTTGAACTGTTTACCCTCCACTATCCTGGAAATAT 360
Genbank_margoi CTTAGTTGAAAATGGGGTAGGTACAGGATGAACCGTGTATCCACCATTATCCAGGTAATAT 359
OMPP1          CTTAGTTGAAAATGGGGTAGGTACAGGATGAACCGTGTATCCACCATTATCCAGGTAATAT 360

OBPP1          ATCAATTTTAGGCTCTATTAATTTCATTACAACAATTATTAATATACGTTGACAAGGAAT 480
OBIRL_consensus ATCTATTTTAGGCTCTATTAATTTCATTACAACAATTATTAATATGCGTTGACAAGGAAT 480
Genbank_margoi ATCTATTTTAGGATCTATTAACTTTATTACAACTATTATTAATATACGATGACAAGGAAT 479
OMPP1          ATCTATTTTAGGATCTATTAACTTTATTACAACTATTATTAATATACGATGACAAGGAAT 480

OBPP1          GCGACTAGAGCGAATGCCTTTATTTGATAGAGCAGTATTAATTACAGTAGTATTGTTACT 540
OBIRL_consensus GCGACTAGAGCGAATACCTTTATTTGTATGAGCAGTACTAATTACAGTAGTATTATTACT 540

OBPP1          TTTATCATTACCTGTATTTGCTGCAGCTATTACAATACTATTAACCTGATCGTAATCTAAA 600
OBIRL_consensus TTTATCATTACCAGTATTTGCTGCAGCTATCACAATACTATTAACCAGATCGTAATCTAAA 600
Genbank_margoi ACTATCATTGCCAGTTTTTGCAGCTGCAATCACAATACTATTAACCAGATCGTAACTTAAA 599
OMPP1          ACTATCATTGCCAGTTTTTGCAGCTGCAATCACAATACTATTAACCAGATCGTAACTTAAA 600

OBPP1          TACTTCCTTCTTTTGACCCTGTAGGGGGCGGTGACCCTGTTTTATATCAGCACCTATTT-- 658
OBIRL_consensus TACTTCCTTTCTTTGACCCTGTAGGAGGTGGTGACCCTGTTTTATATCAGCATCTATTT-- 658
Genbank_margoi TACTTCCTTTTTTTGACCCTGTTGGAGGTGGTGACCCAGTATTGTATCAACACTCTATTTTG 660
OMPP1          TACTTCCTTTTTTTGACCCTGTTGGAGGTGGTGACCCAGTATTGTATCAACACTTATTTTG 660

OBPP1          ----------
OBIRL_consensus ----------
Genbank_margoi ----------
OMPP1          ATTTTTTTGTATCAACACTTATTT 684
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Legend
**O. branchiatus* from the St. Lucie Nuclear Power Plant (OBPP1)
**O. branchiatus* from Indian River Lagoon (OBIRL_consensus)
**O. margoi* from GenBank (Genbank_margoi)
**O. margoi* from the St. Lucie Nuclear Power Plant (OMPP1)

Results and Conclusions

The successful identification of the *O. margoi* leeches from the power plant (OMPP1) verified the effectiveness of our DNA extraction and purification protocol and PCR procedure. The last 24 nucleotides in our DNA sequence are not present in the GenBank *margoi* sequence (obtained from leeches on loggerheads at Virginia Beach, Virginia), suggesting the primers were attached at a different location in the chromosome. Our OMPP1 chromatogram strongly indicates an additional thymine (T) should be present at the beginning of the genetic sequence (four T's instead of three T's). We submitted our *margoi* sequence to GenBank because the sampled leeches were found on loggerheads at a different location (GU985467).

All leeches from Indian River Lagoon belong to the same leech species, because they all shared the same DNA sequence (OBIRL_consensus). The Indian River Lagoon leeches were morphologically identified as *O. branchiatus* and did not have a match in GenBank, which further indicates the leeches are indeed *O. branchiatus* since no genetic record of *O. branchiatus* existed yet in the database. Unexpectedly, *O. branchiatus* leeches were also found on a loggerhead sea turtle from the power plant (OBPP1). There was a small but significant variance between the DNA sequence of these *O. branchiatus* leeches and those found on green sea turtles from Indian River Lagoon. As a result, we added the *O. branchiatus* leech as a new species to GenBank using two different DNA sequences: OBIRL_consensus (GU985465) and OBPP1 (GU985466).

If *O. branchiatus* is the mechanical vector behind FP plaguing sea turtles, then the fact the *O. branchiatus* leech has changed specificity serves as an indication as to why the panzootic affecting mainly *C. mydas* initially has now appeared to a lesser degree in other turtle species. It is important to note, the origins of turtles migrating to Florida are largely unknown, but those captured at the power plant were originally from the sea and probably did not spent significant amount of time foraging in areas with high documented FP cases, such as Indian River Lagoon. This could explain why all turtles at Indian River Lagoon had detectable cases of FP, but none from the power plant site.

Acknowledgements

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