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Introduction

Fibropapillomatosis (FP) is a neoplastic disease originally identified only on green sea turtles (*Chelonia mydas*). The disease is likely to be terminal if tumors are developed internally, but external tumors on the eyes, mouth, and flippers can also lead to fatal impairment of vision and difficulty feeding and swimming. The involvement of an environmental cofactor appears possible since many FP outbreaks occur at sites of poor water quality in Florida, Hawaii, Brazil, and other similar places around the world, but outbreaks have also been recorded at less contaminated sites. Studies have shown an association between FP and the fibropapilloma-associated turtle herpesvirus (FPTHV), but not all turtles with FPTHV develop FP. Thus, the etiological agent of FP is still unknown. Recently, high viral loads of FPTHV were detected in marine turtle leeches (*Ozobranchus* spp.) from a green sea turtle but the study failed to identify the species of marine leech. Leeches, known to be indicators of environmental stress factors, may transmit or activate FPTHV but are impossible to identify at all life stages using current standard taxonomic practices. In this study, character-based DNA barcoding using mitochondrial cytochrome *c* oxidase I (COI) gene as a molecular marker was employed successfully to identify both species of *Ozobranchus* spp. (*Ozobranchus branchiatus* and *Ozobranchus margoi*) at all stages of development from eight different sites in Florida (Daytona Beach to Key West). This is the first study to document the *O. branchiatus* leech on a loggerhead (*Caretta caretta*) and the first to identify multiple leech species infestation on one turtle (*C. mydas*). Genetic sequences for *O. branchiatus* and *O. margoi* were submitted to the National Center for Biotechnology Information GenBank with *O. branchiatus* added as a new species to the database. The spread of FP to other species of turtles combined with the discovery of a new turtle host for the *O. branchiatus* leech suggests the vector organism involvement behind FP maybe species specific.

Methods

❖ Leech specimens were collected in the field directly from *C. caretta* and *C. mydas* turtles captured by net or rescued. The host species was recorded at the time of collection.

❖ Each leech was examined with a microscope and assigned a morphological identity based upon the number of branchidae (gills) on each side of the abdomen; *O. branchiatus* has seven pairs, while *O. margoi* has five pairs.

❖ Genomic DNA was extracted from individual and combined specimens with a Qiagen DNeasy Blood and Tissue kit (Qiagen, Inc. Valencia, California, USA). Additional materials used were proteinase K (Qiagen, Inc.), RNase (Qiagen, Inc.), and ethanol.

❖ Mitochondrial COI sequences (658 bp) were amplified using the Universal Folmer primers (Folmer *et al*. 1994) synthesized by Invitrogen Corporation (Carlsbad, CA, USA):

Forward: LCO 1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3')

Reverse: HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3')

The PCR reaction solutions contained 25 µL AmpliTaq Gold 360 (Applied Biosystems, Foster City, CA, USA), 5 µL of a 2 µM solution of each primer, 70 to 100 ng of DNA template and enough distilled, deionized water for a total volume of 50 µL.

❖ The PCR thermal regime for amplification was 10 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 50 °C, 60 s at 72 °C, and a final elongation of 7 min at 72 °C using an Applied Biosystems PCR System 2700 thermocycler (Singapore).

❖ Purification of PCR products was performed using a QIAquick PCR Purification Kit (Qiagen, Inc.). Amplification products were sequenced in both directions by Retrogen, Inc. (San Diego, California, USA).

❖ Alignment analysis of nucleic acid consensus sequences was done using ClustalW2 (EMBL-EBI). The invertebrate mitochondrial genetic code was used with EMBOSS Transeq (EMBL-EBI) for translating DNA sequences to amino acid sequences (start codon at second position). DNA sequences were submitted to NCBI GenBank for selected samples.

❖ The DNA Barcode was elucidated by comparing the COI sequences of our samples to closely related species following alignment in MEGA 5 Beta. Simple characteristic attributes (sCAs) for the Ozobranchidae family were identified by selecting nucleotide positions with unique characters pertaining to *O.branchiatus* (found on *C. mydas* or *C. caretta*) and *O. margoi*. The COI genetic sequence of cocoon residues obtained from the carapace of the *C. mydas* and the *C. caretta* were compared to the DNA Barcode to determine the species of leech from which they originated.

Species/Abbrv	
1. B. lobata	ATAAGATTTATTATTCGATCAGAGTTATCTCAACCAGGGAGGTTGCTTGAAAATG
2. B. parkeri	ATAAGATTTATTATTCGATCTGAATTATCTCAGCCAGGTAGTTTACTTGAAAATG
3. C. lophii	ATAAGATTTATTATTCGATCTGAATTAAATCAACCAGGAAGATTTATTGATAATG
4. J. arctica	ATAAGATTTATTATTCGATCTGAATTAACCCAACCAGGCAGGTTAATTGATAATG
5. O. typica 2007	ATAAGATTTATTATTCGAACAGAATTATCTCAACCAGGTAGATTAATCGATAATG
6. A. bilobata	ATAAGACTAATTATTCGAGCTGAACTATCTCAACCTGGTAGATTAATTGGAAATG
7. M. lugubris HI	ATAAGAATAATTATTCGAACTGAATTATCTCAACCAGGTAGATTGCTAGAAAATG
8. G. complanata	ATAAGAATTATTATTCGAATTGAGTTAGCTCAACCAGGATCATTTTTAAATAATG
9. A. translucens	ATAAGTCTTATTATTCGAGCTGAACTATCTCAACCAGGTAGTTTAATTGGAAATG
10. B. torpedinis	ATAAGATTTATCATTCGATCTGAACTTTCACAACCAGGGAGATTACTTGAAAATG
11. M. lugubris FW	ATAAGAATAATTATTCGAACTGAATTATCTCAACCAGGTAGATTGCTAGAAAATG
12. O. branchiatus Cc	TTAAGAGTAATTATTCGATTAGAATTAAACCAACCAGGATCATTTTTAAATAACG
13. O. branchiatus Cm	TTAAGAGTAATTATTCGATTAGAATTAAACCAACCAGGATCATTTTTAAATAATG
14. O. margoi Cc	CTGAGAGTTATCATCCGATTGGAATTAAATCAGCCAGGATCTTTCCTGAATAATG

↑ 50 ↑ 52 ↑ 56 ↑ 64 ↑ 70 ↑ 94 ↑ 97 ↑ 103

Figure 1
Multiple sequence alignment of Ozobranchidae leeches and 11 most closely related marine leeches at 50-104 bp region. Positions of unique CAs indicated by arrows.

Taxa	16	25	45	47	48	50	52	56	64	70	94	97	103	112	119	122	130	139	140	163	166	181	187	241	266	290	292
Cocoon residue (CC)	A	T	T	A	G	C	G	G	C	G	C	G	T	G	A	A	C	C	T	A	G	T	C	T	A	G	A
Ozobranchus margoi (3)	A	T	T	A	G	C	G	G	C	G	C	G	T	G	A	A	C	C	T	A	G	T	C	T	A	G	A
Ozobranchus branchiatus-CC/CM (2/16)	A	C	T	A	G	T	A	G	T	A	T	A	C/T	G	A	G	A	T	T/C	G/A	A	A	C	G	A	A	T/C
Cocoon residue (CM)	A	C	T	A	G	T	T	C	T	A	T	N	T	G	A	G	G	N	N	A	A	A	C	G	A	A	C

Taxa	316	319	334	337	343	354	361	367	370	394	445	481	482	483	485	518	535	538	550	571	596	625	628	643	649	653
Cocoon residue (CC)	G	A	C	G	A	G	T	T	T	C	T	A	C	G	T	T	A	T	G	C	T	A	T	G	A	T
Ozobranchus margoi (3)	G	A	C	G	A	G	T	T	T	C	T	A	C	G	T	T	A	T	G	C	T	A	T	G	A	T
Ozobranchus branchiatus-CC/CM (2/16)	T	C/T	T	T	C/T	G	T	C	A/G	T	C	G	C	G	C	T/C	G/A	A	A	T/C	C	G/A	C/T	A	G	C
Cocoon residue (CM)	T	T	T	N	T	G	T	C	A	T	C	G	C	T	C	C	A	A	A	G	C	A	C	A	A	C

Table 2
Nucleotide positions were selected only if unique characters exist pertaining to the Ozobranchidae family. Pure diagnostic characters that are found solely in the Ozobranchidae family are shaded in gray. Any nucleic ambiguity or absence of nucleotide at a given position is represent by a 'N'. At certain positions, pure characters occur in only some members of a species, also known as private characters. Numbers in parenthesis indicate the number of specimens analyzed.

Results and Conclusions

Genetic sequencing of leeches from eight Florida sites (Table 1, Images) reveals two different haplotypes for *O.branchiatus* that were dependent on turtle host species (Table2). *O. margoi* leeches share identical sequences regardless of turtle hosts. Since this is the only study to ever document *O. branchiatus* leeches on a loggerhead, it is still uncertain whether a new haplotype has been identified for the leech species or instead the discovery of a cryptic species. More samples of *O. branchiatus* on loggerhead turtles must be collected and sequenced for multiple genes in order to shed greater insight on these two possibilities.

The need to further study *Ozobranchus* spp. has increased in recent years because of its connection to sea turtle conservation. If *O. spp* is indeed a mechanical vector behind FP transmission/activation among sea turtle populations around the world, then it is imperative that a system of identification be implemented to unambiguously identify these organisms (Williams *et al*. 2006). This study has established such a system .

DNA barcodes (Figure 1, Table 2) for two species of Ozobranchidae leech (McGowin *et al*. 2010) has shown it can be applied successfully to species identification even when morphological taxonomy cannot be employed (e.g. cocoon residues and leeches in the larval stage). Character-based DNA barcoding provides an ideal means of species identification that is in line with current standard taxonomic practices. The criteria for an effective DNA barcode requires that it incorporate genetics, morphology, species behavior, geographic information, and other valid species delimitation attributes (DeSalle 2006).

Although the character-based genetic barcode of Florida leeches yielded a significant number of CAs unique to the Ozobranchidae family (vital to species differentiation), future research should include samples further south of the Florida peninsula (e.g. Lake Worth) and other places within the realm of marine turtle habitats, such as Hawaii, Australia, Brazil, and other Caribbean islands.

References

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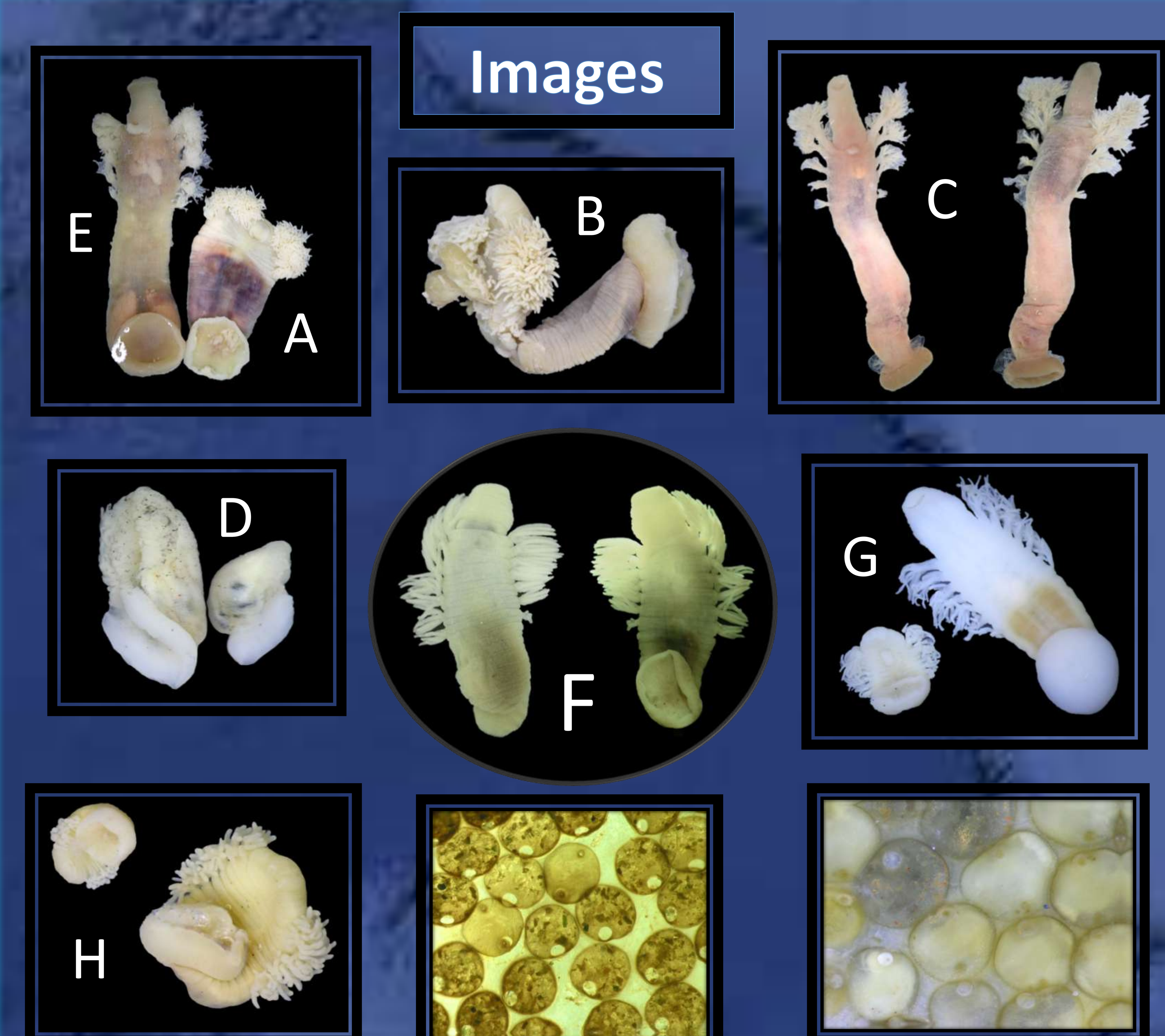
Site	Locations	Species Designation	Host	Haplotype designation	Collection Date (# samples)	GenBank #
A	St. Johns County Intracoastal	*Ozobranchus margoi*	*Caretta caretta*		8/4/2010 (1)	GU985467
B	Daytona Beach	*Ozobranchus margoi*	*Caretta caretta*		4/14/2010 (1)	GU985467
C	Beach Street, Ponce Inlet Ocean Side	*Ozobranchus margoi*	*Caretta caretta*		5/11/2010 (3)	GU985467
D	Indian River Lagoon	*Ozobranchus branchiatus*	*Chelonia mydas*	OB-CM	8/12/2009 (3) 12/07/2009 (12)‡	GU985465
E	Vero Beach	*Ozobranchus margoi*	*Caretta caretta*		7/03/2010 (2)	GU985467
F	St. Lucie Hutchinson Island	*Ozobranchus branchiatus*	*Chelonia mydas*	OB-CM	3/21/2010 (2) 3/23/2010 (2)§	GU985465
			Caretta caretta	OB-CC*	8/25/2009 (2)‡	GU985466
		Ozobranchus margoi	*Chelonia mydas*		3/23/2010 (1)§‡	HM590711†
			Caretta caretta		8/13/2009 (2)‡ 9/15/2009 (1) 3/24/2010 (2)	GU985467†
G	Grassy Key MM 57	*Ozobranchus branchiatus*	*Chelonia mydas*	OB-CM	11/14/2010 (2)	GU985465
H	Barracouta, Key West	*Ozobranchus branchiatus*	*Chelonia mydas*	OB-CM	5/10/2010 (4)	GU985465

Table 1
Ozobranchus branchiatus (OB), *Ozobranchus margoi* (OM), *Caretta caretta* (CC), *Chelonia mydas* (CM)
*Potentially a cryptic species and not a new haplotype.
§Collection dates are identical because leech specimens were collected from the same individual sea turtle.
†Accession number is different due to the fact leeches were found on different hosts and not based upon any genetic variance or haplotype difference.
‡ Leech(es) from the collection date provided the original genetic sequence for GenBank submission.

Images



Chelonia mydas
Cocoon residue

Caretta caretta
Cocoon residue

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