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## DNA barcoding of sea turtle leeches (Ozobranchus spp.) in Florida coastal waters

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# DNA barcoding of sea turtle leeches (*Ozobranchus* spp.) in Florida coastal waters

## WRIGHT STATE UNIVERSITY

### Introduction

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 branchiatus and 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

## Methods

specific.

- \*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. Cenomic 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).

determine the species of leech from which they originated.

- 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

## 

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

insight on these two possibilities. (DeSalle 2006).

# References

| Species/Abbrv        | _   |    | *  |          | *   |   |            |   |   | * | ÷ | ŀ | •   | •  |   | * | * |   |   |            | *  · | *   |   |
|----------------------|-----|----|----|----------|-----|---|------------|---|---|---|---|---|-----|----|---|---|---|---|---|------------|------|-----|---|
| 1. B. lobata         | 2   | A. | TZ | A Z      | A G | A | T          | T | Т | A | T | T | AI  | ΓT | C | G | A | Т | С | A          | G    | AC  |   |
| 2. B. parkeri        | 2   | A  | T  | A.Z      | A G | A | T          | Т | Т | A | T | T | A 1 | ГТ | C | G | A | Т | С | Т          | G I  | A.3 | 1 |
| 3. C. lophii         | 2   | A  | T  | A Z      | AG  | A | T          | Т | Т | A | T | T | AI  | ГТ | c | G | A | Т | С | T          | GI   | A.2 | 1 |
| 4. J. arctica        | 2   | A  | T  | A Z      | A G | A | Т          | Т | Т | A | T | T | AI  | ГТ | C | G | A | Т | С | T          | G I  | A 7 | 1 |
| 5. O. typica 2007    | 2   | A  | T  | A Z      | A G | A | T          | Т | Т | A | T | T | A 1 | ГТ | С | G | A | A | C | A          | G I  | A 2 | Ľ |
| 6. A. bilobata       | 2   | A  | T  | A Z      | A G | A | C          | Т | A | A | T | T | AI  | T  | C | G | A | G | С | T          | G    | A 2 | 1 |
| 7. M. lugubris HI    | 2   | A  | T  | A Z      | AG  | A | A          | Т | A | A | T | T | AI  | гт | c | G | A | A | C | T          | G    | A.2 | 1 |
| 8. G. complanata     | 2   | A  | T  | A.Z      | A G | A | A          | Т | Т | A | T | T | AI  | ΓT | c | G | A | A | T | T          | GI   | A   | ; |
| 9. A. translucens    | 2   | A  | T  | A.Z      | A G | T | C          | Т | Т | A | T | T | A 1 | ГТ | С | G | A | G | С | T          | G    | A Z | 1 |
| 10. B. torpedinis    | 2   | A  | T  | A Z      | A G | A | T          | Т | Т | A | T | C | AI  | ΓT | C | G | A | Т | С | T          | GI   | A Z | 1 |
| 11. M. lugubris FW   | 2   | A  | T  | A Z      | A G | A | A          | T | A | A | T | T | AI  | ГТ | C | G | A | A | С | T          | G I  | A 7 | L |
| 12. O. branchiatus C | c i | Ľ  | T  | A.Z      | A G | A | G          | Т | A | A | T | T | A 1 | ГТ | C | G | A | Т | Т | A          | GI   | A.) | Ľ |
| 13. O. branchiatus C | m   | r  | T  | A Z      | AG  | A | G          | Т | A | A | T | T | AI  | ГТ | c | G | A | Т | T | A          | G    | AZ  | 1 |
| 14. O. margoi Cc     | (   | C  | T  | G I      | AG  | A | G          | T | Т | A | T | c | AI  | C  | c | G | A | Т | Т | G          | GI   | AZ  | 1 |
|                      |     | 1  |    | $\wedge$ |     |   | $\uparrow$ |   |   |   |   |   |     |    |   |   |   |   |   | $\uparrow$ |      |     |   |

### Figure 1

Multiple sequence alignment of Ozobranchidae leeches and 11 most closely related marine leeches at 50 52 50-104 bp region. Positions of unique CAs indicated by arrows

Position 444 55 65 578 0264 0470 Taxa Cocoonresidue (CC) GCG Ozobranchus margoi(3) GCG CGGC Ozobranchus branchiatus-CC/CM(2/16) A T A ( TAG TAGT Cocoonresidue (CM) AC TAG ΤΤΟΤ ATNI Taxa Cocoonresidue (CC) СТА GΑ GTTT CGA Ozobranchus margoi(3) CGA GTTT СТА Ozobranchus branchiatus-CC/CM(2/16) TC/T TTC/T GTCA/G TCG Cocoonresidue (CM) TT TNT GTCA TCGC

#### 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.

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## **Results and Conclusions**

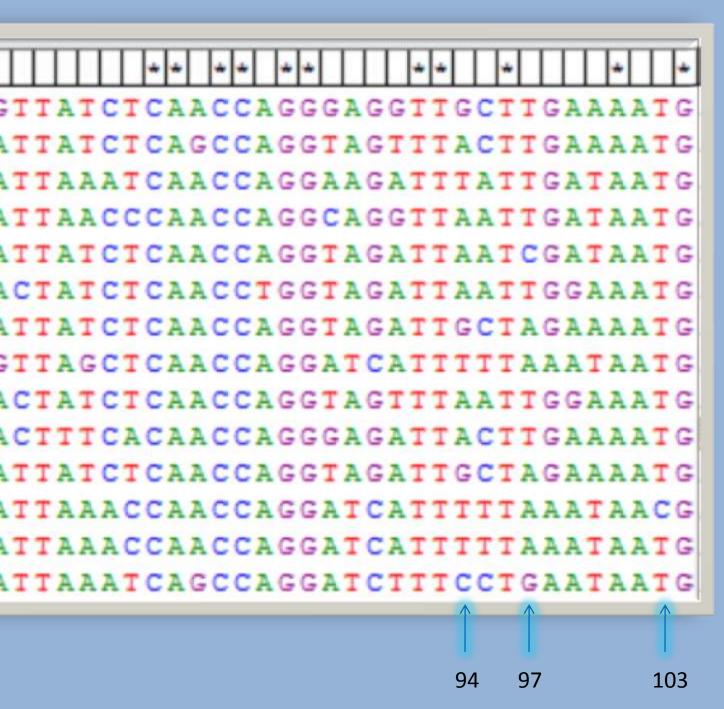
Genetic sequencing of leeches from eight Florida sites (Table 1, Images) reveals two different haplotypes for O.branchiatus that were dependent on turtle host species (Table2). O. margoi leeches share identical sequences regardless of turtle hosts. Since this is the only study to ever document O. branchiatus 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

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

dentification 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

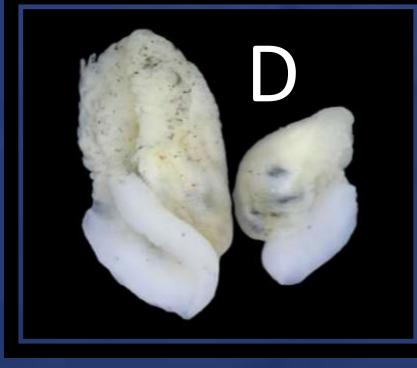
Although the character-based genetic barcode of Florida leeches yielded a significant number of CAs unique to the Ozobranchidae family (vital to species differentiation), future research should include samples further south of the Florida peninsula (e.g. Lake Worth) and other places within the realm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

DeSalle R (2006) Species discovery versus species identification in DNA barcoding efforts: response to Rubinoff. *Conservation Biology*, 20, 1545–1547. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3, 294-299. McGowin AE, Truong TM, Corbett AM, Bagley DA, Ehrhart LM, Bresette MJ, Weege ST, Clark D. (2011) Genetic barcoding of marine leeches (Ozobranchus spp.) from Florida sea turtles and their divergence in host specificity. *Molecular Ecology Resources*, 11, 271–278. Williams EH, Bunkley-Williams L (2006) Early fibropapillomas in Hawaii and occurrences in all sea turtles species: the panzootic, associated leeches wideranging on sea turtles, and species of study leeches should be identified. *Journal of Virology*, 80, 4643–4644.



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|   | 4<br>8<br>3      | 4<br>8<br>5      | 5<br>1<br>8        | 5<br>3<br>5        | 5<br>3<br>8      | 5<br>5<br>0        | 5<br>7<br>1        | 5<br>9<br>6      | 6<br>2<br>5        | 6<br>2<br>8        | 6<br>4<br>3      | 6<br>4<br>9      | 6<br>5<br>3           |   |
|   | G<br>G<br>G<br>T | T<br>T<br>C<br>C | T<br>T<br>T/C<br>C | A<br>A<br>G/A<br>A | T<br>T<br>A<br>A | G<br>G<br>A<br>A   | C<br>C<br>T/C<br>G | T<br>T<br>C<br>C | A<br>A<br>G/A<br>A | T<br>T<br>C/T<br>C | G<br>G<br>A<br>A | A<br>A<br>G<br>A | T<br>T<br>C<br>C      |   |



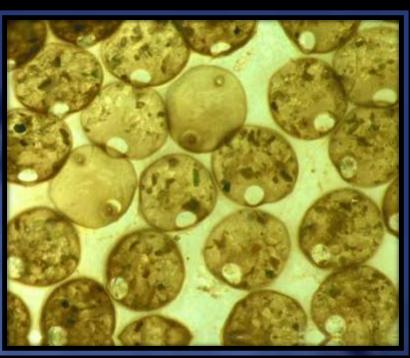




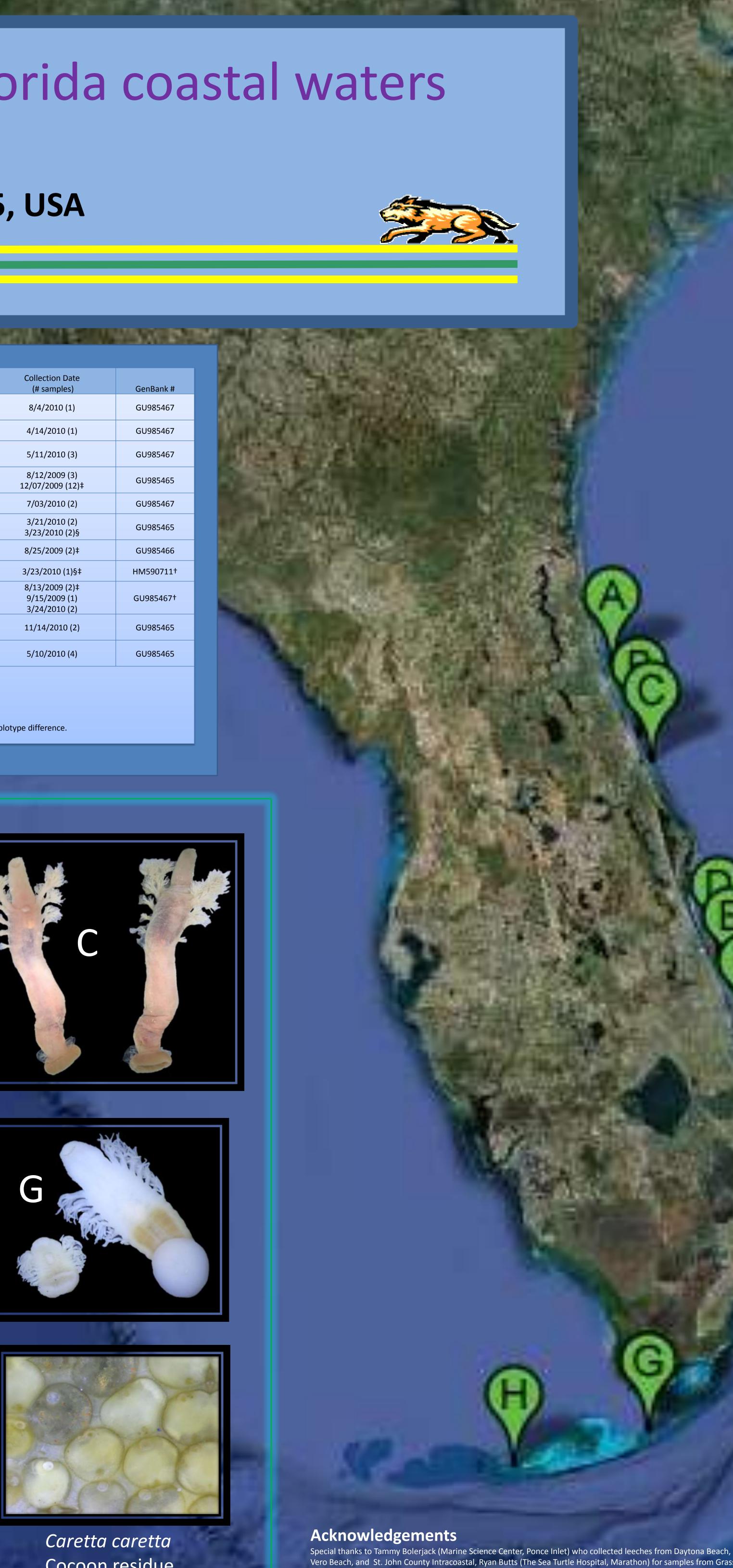
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|----|---|-------------------------|-----------------|-----------------------|--|-----------------------|
| :e | Locations                               | Species Designation     | Host            | Haplotype designation | Collection Date<br>(# samples)                   | GenBank #             |
|    | St. Johns County Intracoastal           | Ozobranchus margoi      | Caretta caretta |                       | 8/4/2010 (1)                                     | GU985467              |
| ,  | Daytona Beach                           | Ozobranchus margoi      | Caretta caretta |                       | 4/14/2010 (1)                                    | GU985467              |
|    | Beach Street, Ponce Inlet<br>Ocean Side | Ozobranchus margoi      | Caretta caretta |                       | 5/11/2010 (3)                                    | GU985467              |
| )  | Indian River Lagoon                     | Ozobranchus branchiatus | Chelonia mydas  | OB-CM                 | 8/12/2009 (3)<br>12/07/2009 (12)‡                | GU985465              |
| :  | Vero Beach                              | Ozobranchus margoi      | Caretta caretta |                       | 7/03/2010 (2)                                    | GU985467              |
|    |   | Ozobranchus branchiatus | Chelonia mydas  | OB-CM                 | 3/21/2010 (2)<br>3/23/2010 (2)§                  | GU985465              |
|    |   | Ozobranchas branchatas  | Caretta caretta | OB-CC*                | 8/25/2009 (2)‡                                   | GU985466              |
|    | St. Lucie Hutchinson Island             |                         | Chelonia mydas  |                       | 3/23/2010 (1)§‡                                  | HM590711 <sup>+</sup> |
|    |   | Ozobranchus margoi      | Caretta caretta |                       | 8/13/2009 (2)‡<br>9/15/2009 (1)<br>3/24/2010 (2) | GU985467†             |
| ì  | Grassy Key MM 57                        | Ozobranchus branchiatus | Chelonia mydas  | OB-CM                 | 11/14/2010 (2)                                   | GU985465              |
| I  | Barracouta, Key West                    | Ozobranchus branchiatus | Chelonia mydas  | OB-CM                 | 5/10/2010 (4)                                    | GU985465              |
|    |   |                         |                 |                       |  |                       |

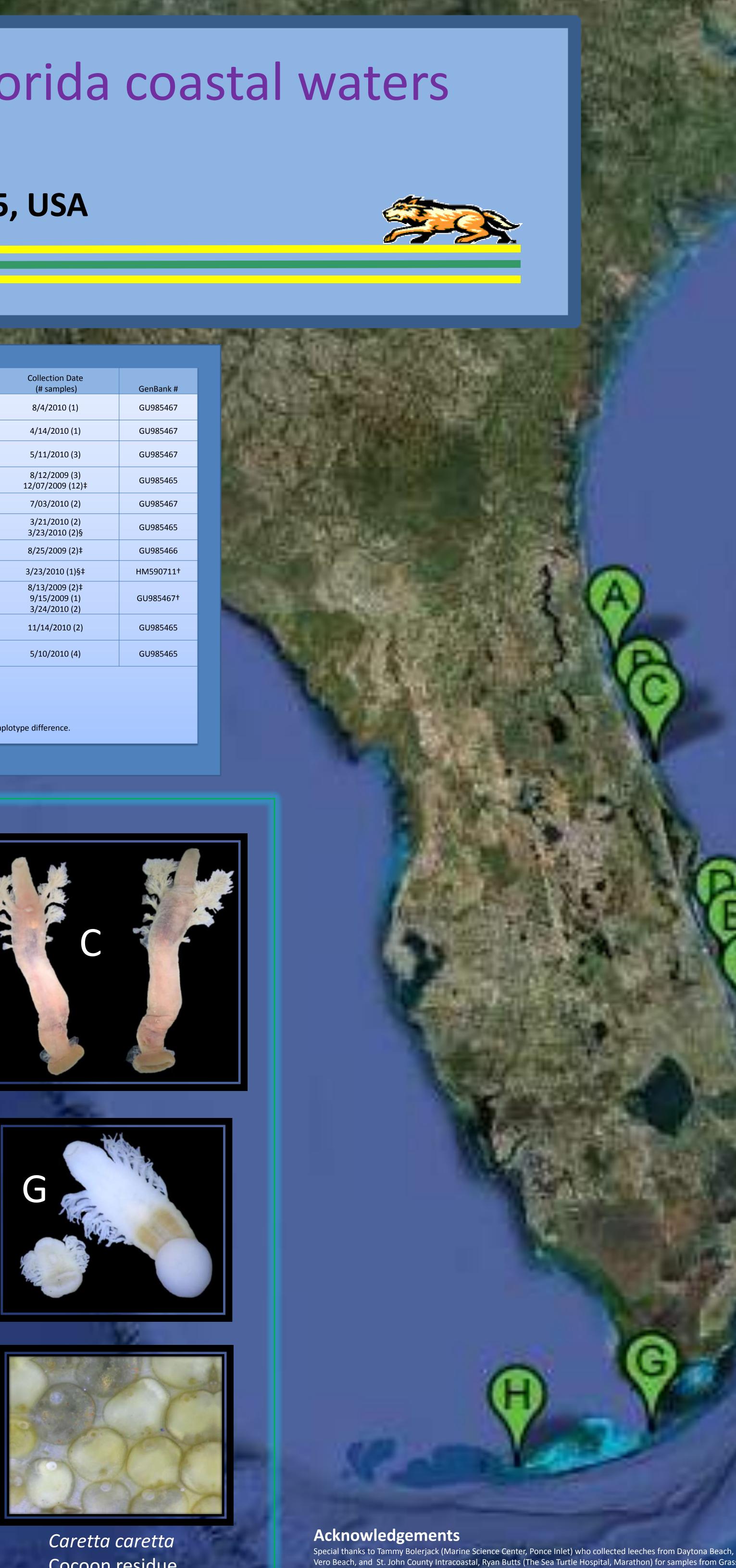






Cocoon residue





Cocoon residue

Special thanks to Tammy Bolerjack (Marine Science Center, Ponce Inlet) who collected leeches from Daytona Beach, Ponce Inlet Vero Beach, and St. John County Intracoastal, Ryan Butts (The Sea Turtle Hospital, Marathon) for samples from Grassy Key, Dean Bagley (University of Central Florida) for leeches from Indian River Lagoon, and Dave Clark and Stephen T. Weege (Inwater Research Group, Inc.) for leeches from the St. Lucie Nuclear Power Plant and Barracouta Key West. John O. Stireman III, PhD and Adrian M. Corbett, PhD (Wright State University) generously provided special equipment and laboratory setting for portions of the research. Dean Rider, PhD provided additional sequencing of leech samples useful for verification purposes.