Extensive mitochondrial CO1 sequence diversity in a population of the freshwater snail, *Physa.*

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Introduction

The most abundant and widespread freshwater snails of North America belong to the pulmonate family *Physidae*. In ponds, lakes, and calm rivers, these snails can indirectly affect whole ecosystems by impacting the amount and composition of algae and aquatic plants by their grazing. Physid snails, in turn, may then become a significant component of the diet of fishes, crayfishes, and other aquatic predators (Dillon 2000).

Because of their adaptability to culture and the cosmopolitan nature of the species, the breeding biology of *Physa acuta* has been of interest recently. Despite numerous studies, the taxonomy of the snail remains in a confused state. It has been reported in many parts of the world including Europe, the Middle East, Africa, North and Central America, and others. In the past, descriptions of many species within the *acuta* group were based solely on minor differences in shell shape or color resulting in the description of many dozens of species, subspecies, and so-called "morphs" (Dillon, et al. 2002)

A recent study by Wethington, et al. currently in preparation has reported that snails from a single population of *P. acuta* at Charles Towne Landing State Park differ at 24-27% of base pairs in their cytocrome c oxidase 1 (CO1) DNA, an unusually high percentage of variation within a single species.

To begin to unravel the nature of this anomaly, DNA was isolated from a random sample of 22 Charles Towne Landing snails. Polymerase Chain Reaction (PCR) and Cycle Sequencing techniques were then used to determine an approximately 650bp fragment of the CO1 gene. Breeding studies are planned to determine whether the high genetic diversity observed by Wethington is better attributed to admixture of two conspecific populations or a pair of cryptic species coexisting.

Methods and Materials

Culture

A total of 26 *P. acuta* of various sizes were collected from Charles Towne Landing State Park in Charleston, SC. Following the culture methods outlined by Dillon (Dillon, et al. 2002), all snails were placed in transparent plastic cups of aerated and filtered pond water (210mL). Disposable Petri-dish lids covered the cups, and snails were fed a green flake fish food (OSI brand) with a spirulina base. All snails were cultered at room temperature (~23ºC) in a 12/12 daylight cycle. Snails were transferred to new cups every 7-10 days as egg masses were laid, and the water was changed until a total of three generations of progeny existed. Of the 26 snails, 22 were designated for the parental generation and each generation was designated ND1.1 followed by ND1.2, etc.

DNA methods

Tissue was isolated from the head and tail of each animal using a scalpel under a stereomicroscope and placed in 95% ethanol for preservation. DNA was extracted using DNAeasy extraction kits (Qiagen). Double-stranded mitochondrial DNA was amplified using Polymerase Chain Reaction (PCR) to create a 650 base pair segment. Using 50-500ng of template genomic DNA each, a total of five PCR reactions of varying dilutions were performed for each sample in 50µL reaction volumes (25µL HSTaq by Qiagen containing Tris, KCl, $MgCl_2$, each dNPT, and Taq DNA polymerase; each primer diluted 10X).

PCR products were then run on an agarose gel (3.0g agarose, 300mL 1X TBE buffer) and stained using ethidium bromide. The gel was then view under a UV lamp to determine whether amplification had occurred.

Positive amplification samples were pooled and purified using QIAquick Spin kits (Qiagen) and samples were taken to the Medical University of South Carolina for Cycle Sequencing. Upstream and downstream strands were then aligned using nucleotide-nucleotide BLAST found on the NCBI website (www.ncbi.nlm.nih.gov). One of the sequences was then transferred to BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) for comparison to all other sequences.

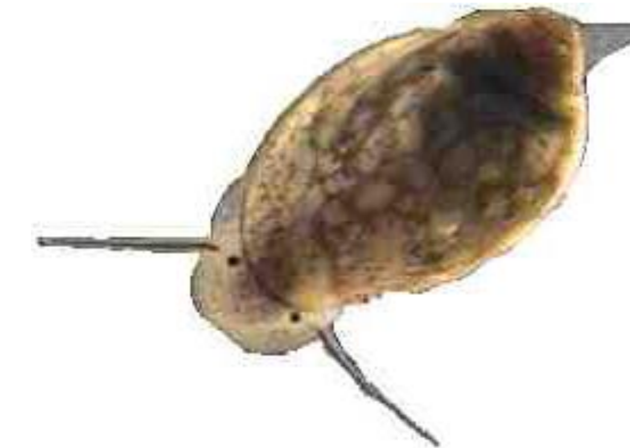
Figure 1: *Physa acuta*.

Figure 2: *P. acuta* cultures

Results

Sequences of six *P. acuta* were aligned with one another and with the 20 DNA sequences obtained by Wethington. A matrix was then created which compared each sequence to all others and returned a score for the percentage of base pairs that two sequences shared (Figure 5). Those with a high percentage of bases (>90%) in common with the more common haplotype observed by Wethington were labeled "Archetypal" and those which matched the rare haplotype were termed "Zany."

Of the six snails sequenced thus far (ND1-ND5 and 25C6), all were archetypal, varying between 87-99% similarity with the more common haplotype observed by Wethington. Most showed greater than 90% shared base pairs with the exception of ND5. Because it showed less than 70% similarities to the "Zany" haplotype, it is assumed that ND5 was simply a bad sequence but otherwise archetypal.



Sequence	ND1	ND2	ND3	ND4	ND5	25C6#	COC10	COC13	COC25	COC29
COC11	0.979	0.927	0.960	0.918	0.874	0.995	0.763	0.760	0.757	0.757
COC12	0.982	0.929	0.960	0.919	0.875	0.998	0.765	0.763	0.760	0.760
COC14	0.998	0.916	0.957	0.907	0.875	0.981	0.762	0.779	0.776	0.776
COC16	0.984	0.930	0.960	0.921	0.877	0.996	0.763	0.765	0.762	0.762
COC20	0.985	0.921	0.960	0.911	0.869	0.984	0.755	0.770	0.766	0.766
COC21	0.993	0.921	0.955	0.911	0.880	0.985	0.766	0.774	0.771	0.771
COC27	0.957	0.891	0.932	0.881	0.851	0.957	0.755	0.740	0.740	0.740
COC2H	0.966	0.891	0.932	0.881	0.853	0.951	0.754	0.765	0.762	0.762
COC3H	0.965	0.900	0.943	0.891	0.853	0.960	0.746	0.759	0.755	0.755
COC4L	0.973	0.922	0.960	0.913	0.870	0.988	0.770	0.757	0.754	0.754
COC5H	0.955	0.894	0.933	0.888	0.858	0.952	0.735	0.746	0.743	0.743
COC6H	0.990	0.924	0.965	0.914	0.870	0.988	0.755	0.773	0.770	0.770
COC9L	0.963	0.899	0.938	0.889	0.861	0.963	0.760	0.748	0.744	0.744
coctl	0.998	0.916	0.957	0.907	0.875	0.981	0.762	0.779	0.776	0.776
coctl	0.979	0.927	0.960	0.918	0.874	0.993	0.763	0.760	0.757	0.757
ND1	1.000	0.916	0.955	0.907	0.875	0.981	0.762	0.777	0.774	0.774
ND2	---	1.000	0.897	0.930	0.874	0.927	0.722	0.722	0.719	0.719
ND3	---	---	1.000	0.888	0.847	0.959	0.738	0.748	0.744	0.744
ND4	---	---	---	1.000	0.881	0.918	0.716	0.716	0.713	0.713
ND5	---	---	---	---	1.000	0.874	0.696	0.692	0.689	0.689
25C6#	---	---	---	---	---	1.000	0.763	0.762	0.759	0.759
COC10	---	---	---	---	---	---	1.000	0.976	0.979	0.979
COC13	---	---	---	---	---	---	---	1.000	0.996	0.996
COC25	---	---	---	---	---	---	---	---	1.000	1.000
COC29	---	---	---	---	---	---	---	---	---	1.000

Figure 5: Matrix of results obtained from BioEdit.

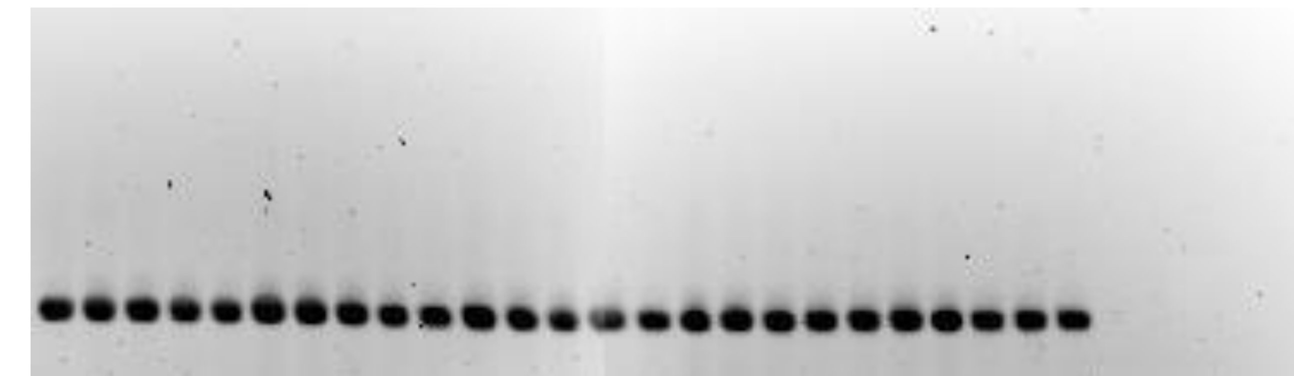


Figure 3: Gel of PCR products showing positive results (ND11.1-ND15.1)

Discussion

Because of the large amount of *P. acuta* sequencing data still to be obtained, it is impossible to tell if the 100% frequency of Archetypal snails is due to a higher than expected ratio of Archetypals to Zanies in nature. The sequencing techniques have thus far proved effective and it is not likely that they will be altered.

Future research involving the lines from the *P. acuta* which have been sequenced will be used to determine positive matches on each of the two haplotypes. Wethington predicts that the likelihood of a pair of coexisting cryptic species is remote and that there will be no reproductive isolation between lines carrying the two haplotypes. Breeding studies involving the two followed by more sequencing should confirm or refute this hypothesis

Acknowledgements

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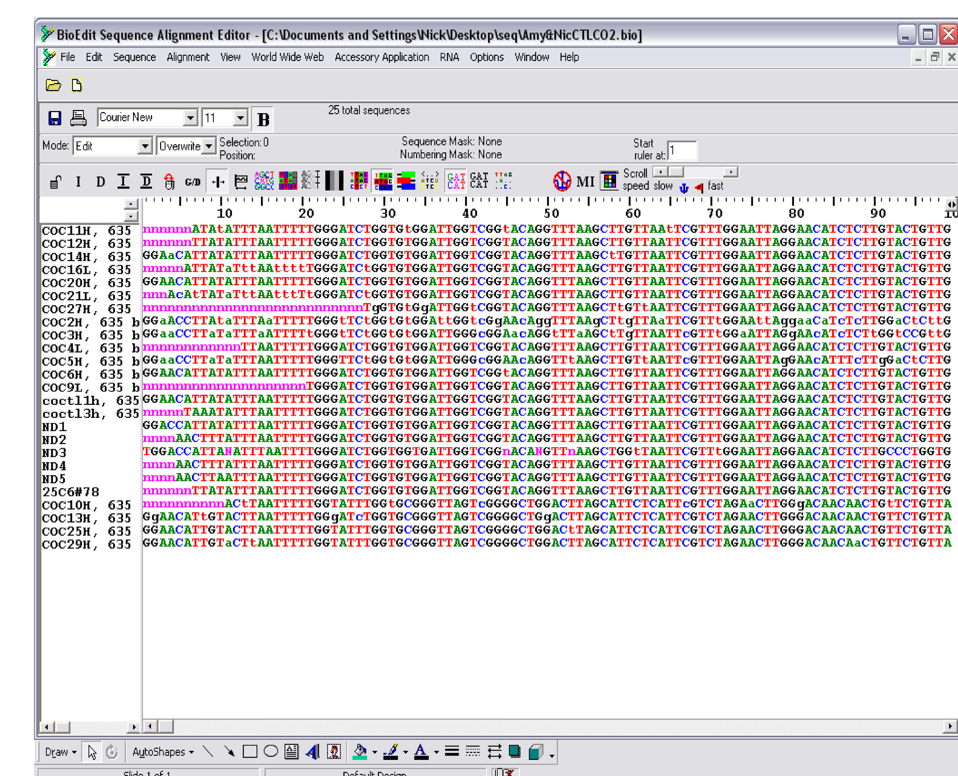

Figure 4: Shot of BioEdit program showing aligned sequences.