

November 4, 2009

Final Report: Genetic Connectivity among populations of the Caribbean Queen Conch (*Strombus gigas*) in Belize, Central America

Submitted by John Cigliano and Richard Kliman (Cedar Crest College, PA)

Workplan Activity Title (3.1.2): Assessment of DNA sequence variation and population structure in queen conch of Belize

Study Site: Sapodilla Cayes Marine Reserve, Belize

A. Objectives as stated in original workplan

The primary objective of this study is to assess levels and patterns of DNA sequence variation in Belize populations of the queen conch, *Strombus gigas*. We aim to determine if there is sufficient genetic polymorphism (*e.g.*, as measured by pairwise variation) and population structure (*e.g.*, as measured by F_{st}) to estimate gene flow. Estimating gene flow requires some degree of population structure and a sufficient number of sampled polymorphic sites in DNA sequences. That is, at a subset of positions in the DNA sequences, there must be some variation among individuals. However, polymorphism is not sufficient to estimate gene flow; there must be sufficient variation among populations (*i.e.*, structure) in the distribution of the polymorphism to differentiate populations from each other.

These results will provide the data needed for subsequent work that will examine if the shallow-water queen conch population in the Sapodilla Cays Marine Reserve is genetically distinct from other populations (*e.g.*, deep-water populations, Port Honduras, Middle and North Mesoamerican Barrier Reef).

Outcomes

Tissue samples were obtained from approximately 50 queen conch and from 7 milk conch (*S. costatus*, a close relative). Sampling was performed in 2006 and 2007 in the Sapodilla Cayes Marine Reserve (SCMR), in 2007 and 2008 at Turneffe Atoll (TA), and in 2007 in the Port Honduras Marine Reserve (PHMR). Prior to this work, tissue was collected in 2003 from five individuals in the Turks and Caicos Islands (TCI).

Prior to 2008, tissue was preserved in isopropyl alcohol; in 2008, separate tissue samples were preserved in isopropyl alcohol and RNAlater (a reagent manufactured by Ambion, Inc.). The switch was a response to problems that were detected when we began to sequence nuclear genes (see below).

Our initial efforts focused on the mitochondrial *COI* gene. This gene is commonly used in conservation genetics research. Being located in mitochondria, it is at high copy number relative to nuclear genes. It is also uniparentally inherited (from mothers), so there is usually no variation within an individual. [Nuclear genes, being biparentally inherited, often exist in two forms, which complicates sequencing efforts.] These two qualities of *COI* lend it well to genetic analysis. Even samples with degraded DNA remain suitable, since the "noise" caused by lesions

in the DNA is swamped out by reliable "signal." We sequenced CO1 in 47 *S. gigas* and 2 *S. costatus*. For pooled *S. gigas*, pairs of individuals differed on average at 0.518% of their sequence positions. When analyses were restricted to conch collected at specific locations, variation within these samples (*i.e.*, "diversity") was comparable to overall variation (see Table 1).

Table 1. DNA sequence variation at CO1 within queen conch samples.

Sample	Sample size	Average pairwise variation
SCMR 2006	10	0.00521
SCMR 2007	8	0.00415
TA 2007	10	0.00616
TA 2008	6	0.00585
PHMR 2007	8	0.00525
TCI 2003	3	0.00474

Related to this finding, average variation among samples (*i.e.*, "divergence") was comparable (see Table 2). F_{ST} values, calculated from divergence and diversity (and an indication of population structure), were all close to zero, with some negative (an outcome that occur because of statistical sampling error). **The data indicate that, at least with respect to CO1, there is no more variation among locations than would be expected on the basis of variation within locations.**

Table 2. DNA sequence divergence at CO1 among queen conch samples.

	SCMR 2007	TA 2007	TA 2008	PHMR 2007	TCI 2003
SCMR 2006	0.00456	0.00559	0.00498	0.00498	0.00482
SCMR 2007		0.00486	0.00504	0.00437	0.00504
TA 2007			0.00600	0.00527	0.00640
TA 2008				0.00543	0.00513
PHMR 2007					0.00553

The most obvious conclusion that could be drawn from these findings is that queen conch have a history of gene flow that obscures any demographic discontinuity that might exist today. That is, one cannot conclude that populations are demographically isolated, although one cannot conclude the opposite. If populations have been exchanging individuals (either bidirectionally or via a stepping-stone model that ultimately connects populations) in the past, this could fully mix genetic variants. Thus, if exchange has slowed or stopped, there would be insufficient time for populations to accumulate "private" genetic variation; therefore, there would be no signal of population structure.

We knew in advance that this result was possible, and we also knew that basing strong conclusions on data from a single gene is inappropriate. For one thing, a history of *recent* admixture could lead to the observed data, but data from additional genes could, in principle, distinguish a model of recent admixture from one of long-standing admixture. This is not to suggest that the data would provide a basis for conservation planning; but they would tell us something about the history of the species. Thus, it is important to add genes to the analysis, and we have continued to work on this challenge.

November 4, 2009

Prior to receiving support from Conservation International, we attempted to characterize nuclear regions by (1) constructing a genomic library for *S. gigas* (*i.e.*, a collection of random DNA fragments), and (2) by constructing a cDNA library for the Florida fighting conch, *S. alatus* (*i.e.*, a collection of random RNA sequences that correspond to protein-coding genes). The genomic library was enriched for microsatellites (a.k.a. short tandem repeats), which are often used in ecological genetic analyses. Unfortunately, the microsatellites were not suitable for population genetic analysis, due to complications caused by flanking sequences. We were, however, able to use the genomic library to identify "anonymous" markers – sequences that could be amplified by the polymerase chain reaction (PCR), cloned into bacteria, and sequenced. Analysis of two such anonymous markers uncovered DNA damage that made analysis of anonymous markers impossible. Because each individual has two copies of each nuclear marker, PCR would amplify both simultaneously. However, damage to the DNA would allow PCR to create products that were randomly recombined parts of one copy with parts of the other. Thus, when we sequenced 20 cloned PCR products from one individual, we obtained 20 distinct sequences – when there should only be two. It was clear that while preservation in isopropyl alcohol was sufficient for sequencing of *COI*, it would not work for nuclear genes. For this reason, we stored samples collected in 2008 in RNAlater. DNA collected from these samples was in considerably better shape.

Our effort to find genes using the *S. alatus* cDNA library were not successful. We did sequence *S. alatus* genes, but we could not amplify these in *S. gigas*. It appears that the evolutionary distance between the two species is too great to design PCR primers for *S. gigas* on the basis of *S. alatus* sequence. In a related effort, we scanned the public DNA sequence database (Genbank) for all published gastropod sequences, and attempted to amplify several of these in *S. gigas*. Again, because the organisms were too distantly related, repeated efforts to design PCR primers proved to be fruitless.

We ultimately decided to use a "brute force" approach to identify nuclear genes in *S. gigas*. Using one of the TA 2008 samples, we tested various approaches to isolate high-quality DNA, with the intent of submitting it for high-throughput genomic DNA sequencing. The first two approaches were unsuccessful – the sequencing facility at Duke University was unable to chop the DNA into sufficiently small fragments. We finally developed a 5-day protocol that yielded DNA of sufficient quality, and were rewarded with ~54,000,000 bases of *S. gigas* DNA sequence. In the spring of 2009, we wrote a computer program to scan these sequences for microsatellites, and settled on four that should be good for population genetic analysis. In the summer of 2009, we began the process of amplifying these markers, and are currently sequencing one of these. We are now preparing to search the *S. gigas* genomic sequences for protein-coding genes, using bioinformatics approaches.

B. Target Audience (globally & site-specific) as stated in original workplan

Dr. Cigliano and Dr. Kliman have identified and engaged the site-specific target audience and discussed how this effort will meet Belizean conservation needs and how this audience could participate. This site-specific audience includes the Belize Department of Fisheries, Toledo

November 4, 2009

Association for Sustainable Tourism and Empowerment (TASTE), University of Belize, and local stakeholders. These organizations have been involved in the initial development of the broad research plan for the SCMR and have been consulted regularly through email and visits (March 2005 and January 2006) to help refine specific research objectives (e.g., genetic connectedness of queen conch populations). The PIs plan to continue to collaborate with these institutions, along with members from the Toledo Institute for Development and Environment (TIDE). Members from each organization (including faculty and students from the University of Belize) have been invited to be part of the field teams to assist in the collection of ecological data on conch populations and to assist in the sampling of conch for genetic analysis. Several students from Natural Resource Management program at the University of Belize have assisted in fieldwork, which was in support of their senior theses.

The PIs have also targeted the international community of conservation scientists and practitioners through professional meetings, including annual meetings of the Society for Conservation Biology, Gulf and Caribbean Fisheries Institute, Ecological Society of America, joint meeting of the Society for the Study Evolution, American Society of Naturalists, and Society of Systematic Biologists, and the 3rd International Conservation Genetics Symposium, and publication of results in peer-reviewed journals. They also hope to have students from the University of Belize assist in the population genetic work as interns at Cedar Crest College.

Furthermore, once the CI point person for Belize has been designated he/she can further engage audiences to assess:

- How the results of this study can contribute to their conservation efforts.
- How they would like to be engaged or participate in the project.
- The products most useful to them and their colleagues in achieving their conservation goals.

CI-MMAS staff will be mainly responsible for engaging target audiences at the global and regional levels. Because the conch fishery is found in all parts of the Caribbean the results from this study are most relevant to countries in this region. The organizations that would form this audience include a mixture of government, regional bodies and environmental institutions. They could include groups like:

Mexican, Guatemalan and Honduran Departments of Fisheries
Meso-American Barrier Reef Systems Project
The Nature Conservancy (marine program)
Caribbean Regional Environmental Program
Gulf and Caribbean Fisheries Institute
Caribbean Fisheries Management Council

Outcomes

We have worked closely with TASTE (and, subsequently, the Southern Environmental Association [SEA]) on a related study of *S. gigas* demographics in the SCMR. We have also shared our findings with various groups of stakeholders, working closely with CI point-person in

November 4, 2009

Belize, Dr. Melanie McField. These have included an open presentation in the summer of 2007 in Punta Gorda and two presentations in the summer of 2009 (one at the fisheries office in Belize City and one at the SEA office in Punta Gorda). These presentations have been attended by fishers, fisheries officials, NGO representatives, and students and faculty from the University of Belize. We also met in January 2009 with a dean and faculty member at the University of Belize to discuss ways to involve students and faculty in ongoing research.

C. In-Country Capacity Building in original workplan

The PI, who is not from Belize, has been selected for his expertise in population genetics. However, we have actively reached out to in-country scientists, as appropriate, through our relationship with the University of Belize, Department of Fisheries, TASTE and TIDE. In addition, we plan to develop case studies and lectures based on the results of this study and share them with our colleagues at the University of Belize. This will be done during a joint field program that is being developed by University of Belize and Cedar Crest College. Additionally, we have discussed our results with local shareholders through community meetings and have included UB students in our field. Contingent upon additional funds, Belizean student interns will work in the PI's lab (June-August) and will learn important molecular biology skills, such as Polymerase Chain Reaction (PCR), DNA electrophoresis, DNA sequencing, and population genetic analysis methods.

Deliverables Intended for the Broader Conservation Community in original workplan

- A final report of the results for CI staff, University Belize collaborators, Belize Department of Fisheries and members of the Toledo Association for Sustainable Tourism and Empowerment (Cigliano and Kliman).
- Education material on queen conch, the Sapodilla Cayes, and marine conservation and management which will be shared with TASTE and TIDE both of which conduct educational outreach. We will also share these materials with any other interested organizations (free of charge) (Cigliano and Kliman).
- We anticipate presentations at the following conferences: Society for Conservation Biology, Society for the Study of Evolution, Belize National Marine Science Symposium, Gulf and Caribbean Research Institute (Cigliano and Kliman).
- Case studies and lectures in marine conservation ecology for courses at University of Belize and Cedar Crest College (JAC). These will be delivered during a joint field course between Cedar Crest College and the University of Belize. During this field course, which will be team-taught by JAC (CCC) and Arlenie Perez (UB), students from both institutions will conduct joint research projects on marine conservation and natural resource management at the SCMR (Cigliano).

Outcomes

As noted earlier, our closest in-country working relationship has been with SEA (formerly as TASTE), the NGO that manages the SCMR. We have an open line of communication, and we

November 4, 2009

share methodologies and data. We also communicate with TIDE, and meet with TIDE representatives when in Punta Gorda. TIDE has helped with sample collection in PHMR.

Efforts to establish a working relationship with the University of Belize have been less successful, mainly because of financial constraints. There does seem to be interest in collaboration, but funding is the issue. For example, in 2007, we applied for support from the National Science Foundation under the "International Research Experience for Students" program. As part of that proposal, we worked out an agreement with UB to include UB students in the research; one student would have spent 6-7 weeks at Cedar Crest working in the lab. Unfortunately, we were unable to secure funding. In 2009, we met with Professor Leandra Chorricketts and Dean Thippichetty Thiagarajan to discuss possible student/faculty collaborations. We hope that these discussions will continue.

Findings of the research have been presented at multiple conferences. These have included the joint meeting of the Society for the Study Evolution, American Society of Naturalists, and Society of Systematic Biologists (summer of 2008 in Minneapolis, MN, USA), the meeting of the Society for Conservation Biology (summer of 2008 in Chattanooga, TN, USA), the Annual Gulf and Caribbean Fisheries Institute Conference (autumn of 2007 in Punta Cana, Dominican Republic), the International Conservation Genetics Symposium (autumn of 2007 in New York City, USA), and the meeting of the Ecological Society of America (summer of 2007 in San Jose, CA, USA). We have shared our data with Dr. Stephen Palumbi (Stanford University), who has assessed *COI* sequence variation in others parts of the Caribbean, and we hope that the data that we continue to collect will lead to peer-reviewed publications on the population genetics and demographics of *S. gigas*.

The joint field course was planned for January 2006. However, the UB faculty member who has been involved went on leave to earn her Ph.D. The field course was still taught (using the Oceanic Society's facilities on Blackbird Caye, Turneffe Atoll). We remain open to the possibility of teaching a joint course.

Educational materials are currently being developed, and will be shared with SEA, TIDE, and others.

FUTURE PLANS

As noted above, we will continue to characterize population genetic variation in queen conch of Belize. The genomic data are an excellent resource; once curated, these will be made publicly available.

From a conservation standpoint, the field work at the SCMR has strong potential to test the effectiveness of a marine reserve. We have four years of pre-enforcement data, and there is strong indication that enforcement of the SCMR will begin soon. This means that a planned before-after-control-impact (BACI) study, supported in the past by Earthwatch, is proceeding as we had hoped. Unfortunately, Earthwatch halted its operation in Belize, which means that we need to identify alternative sources of funding for this important project.