I. Project Title: Invasive seaweed population genetics and reproduction  
(Acanthophora spicifera)  
Principle Investigator: Dr. Alison Sherwood  
Project Staff: Daniel O’Doherty (MS graduate student)  
Organization: University of Hawaii – Botany Department  
Grant #: NA05NOS4261157  
Date: 31 January 2007

II. Executive Summary  
- Acanthophora spicifera field collections were made from a variety of different habitats on Kaua‘i, O‘ahu, Moloka‘i, Maui, and Hawai‘i reefs  
- Reproductive status was determined for all populations collected  
- Developed 7 novel high-resolution genetic markers (microsatellites)  
- DNA extracts made from field-collected individuals and subjected to genetic analysis at 10 different loci: 7 microsatellite markers, one nuclear ribosomal RNA marker, one mitochondrial spacer marker, and one plastid ribosomal RNA marker  
- Results from genetic analyses used to determine presence and nature of genetic structure (e.g. relationships within and among populations from throughout the Hawaiian Islands)  
- Genetic results of microsatellite markers used to estimate the occurrence and frequency of asexual clonal reproduction  
- Substantial degree of variation observed in genetic data that suggested multiple or ongoing introductions of A. spicifera into the state  
- Co-operation with resource managers:  
  - microsatellite protocol allows inexpensive ($1.50 per sample) analysis and identification of possible sources for newly discovered populations  
  - reproductive and genetic data provide several criteria for resource managers to target populations for effective control efforts (targeted removal efforts are currently – as of January 2007 - being organized by Daniel O’Doherty, Ryan Okano, Drs. Robert Nishimoto and Alison Sherwood)

III. Purpose  
A. Detailed description of the resource management problems to be addressed.  
Worldwide algal invasions are associated with alterations of ecosystem structure, reduction of biodiversity, and even catastrophic collapses of coral reef ecosystems. Acanthophora spicifera is the undoubtedly the most widespread invasive alien macroalga in Hawai‘i. This species thrives in an extremely wide range of sub- and inter-tidal environmental conditions and colonizes a variety of natural and artificial substrata throughout the Main Hawaiian Islands (MHI). Previous research has documented the ability of A. spicifera to attach to, and compete with native Hawaiian algal species. This species occupies up to 50% of the substrata on some Hawaiian reefs. Fragmentation, re-attachment, and re-growth is believed to be responsible for a substantial amount of the standing crop for this species. In addition to asexual reproduction by fragmentation, A. spicifera is the only invasive seaweed regularly observed in a sexually reproductive state.
Sexual recombination is likely to result in increased genetic diversity. Genetic variation and selection are often associated with high fitness and adaptability. Because *A. spicifera*, as opposed to other invasive seaweeds, occupies a wider range of habitats and is sexually reproducing, it is likely to have substantial negative impacts and represents a priority species for resource managers.

**B. Detailed description of the questions asked to answer the resource management problems.**

Very little research has been conducted regarding the dispersal and genetic structure of algal populations, including those of alien, invasive species. For resource managers to make informed decisions and respond to future algal invasions, the following basic questions regarding mechanisms of spread and dispersal must be answered.

1. Are populations of *A. spicifera* changing in genetic structure as they disperse throughout the islands?
2. Do the patterns in the genetic data support a single introduction of this species to the state, or multiple, possibly ongoing introductions?
3. Are different reproductive strategies associated with particular patterns in genetic variation?

**C. Objectives to answer each question.**

1 & 2. Use molecular analyses of microsatellite and DNA sequence markers to assess degree and patterns of genetic structure in Hawaiian populations of *A. spicifera*.
3. Examine and record reproductive status of Hawaiian collections of *A. spicifera* to analyses along with patterns of genetic variation.

**IV. Approach**

**A. List of individuals and organizations actually performing the work**

1. Daniel C. O’Doherty (Research Assistant), University of Hawai’i Botany Dept.:
   - **Field Collections, Lab Technician, data analysis and interpretation:**
     - Development of custom DNA extraction protocol - For algal taxa, the extraction of large quantities of relatively pure, high molecular weight DNA necessary for microsatellite marker development is known to be notoriously difficult; development of a suitable technique required testing and modification of four different DNA extraction protocols.
     - Sample collection and preservation
     - DNA extraction
     - Preparation of voucher specimens
     - Testing, troubleshooting, and optimization of PCR amplification of target genetic loci
     - Programming of genotyping software to interpret microsatellite raw data
     - Screened microsatellite loci
     - Conduct large-scale data collection
     - Application of statistical tests to detect asexual reproduction and genetic structure

2. Dr. Alison Sherwood (PI), University of Hawai’i Botany Dept.
   - HCRI presentation – January 2006
   - construction of website
3. Genetic Identification Services

**Contracted to develop microsatellite loci:** January 2006 – May 2006

GIS contracted to develop microsatellite loci for several motifs. Microsatellite primers have been developed for fewer than ten algal species; in algae, microsatellite loci occur less frequently than in animal or vascular plant taxa, and also tend to exhibit less polymorphism. GIS estimated production of PCR primers by April 2006, but because of the aforementioned characteristics of algal DNA, primers were not received until late May 2006.

B. Material list

- **Field collections:** snorkel equipment, collection bags, silica gel
- **Microscopes and voucher preservation:** microscope slides, herbarium paper, formalin, and scintillation vials
- **DNA extraction materials:** micropesles and tubes, CTAB buffer, chloroform, mercaptoethanol, RNAse, Qiagen DNeasy Plant Mini-Kits, isopropanol, ethanol
- **PCR consumables:** PCR reagents, microtubes, 96-well plates, sterile adhesives
- **Electrophoresis:** electrophoresis gel boxes, UV lamp, gel documentation system, buffers, agarose gel, loading dye, molecular ladders
- **Thermalcycler, DNA sequencer / fragment analyzer**
- **Multiplex genotyping:** fluorescently-labeled primers
- **Genemarker:** software for interpreting microsatellite raw data

C. Construction instructions for anything used to accomplish the IIIC objectives

- no construction needed

D. Deployment steps

- no deployment

E. Data collection procedures:

1. Field collections:
   Specimens were collected from subtidal and intertidal habitats by snorkeling or wading. At least two collections were made from each island: Kaua‘i, O‘ahu, Moloka‘i, Maui, and Hawai‘i (Appendix, page 1). Forty individuals were collected from an area no more than 30 meters on the longest dimension for each locality. Upon collection, individual thalli of *A. spicifera* were packaged in plastic bags with a generous amount of clean seawater. Samples were transported back to the laboratory in a cooler for examination and processing for DNA extraction.

2. Determination of reproductive status:
   Each individual alga was examined under stereomicroscopy to determine and record reproductive status: tetrasporangial, spermatangial, or carposporangial.

3. DNA extraction:
   DNA used for genetic analyses was extracted using a modified CTAB protocol or the Qiagen DNeasy Plant Mini-kits.
   The CTAB method involved incubating the lysed algal sample with 400 µL of 2% CTAB buffer containing 4 uL mercaptoethanol. The aqueous phase was extracted twice with 500 uL chloroform and precipitated with an equal volume of 100% isopropanol. A DNA pellet was collected by centrifugation of the aqueous solution for 15 minutes at 8,000 rpm The DNA was washed twice with 500 µL 80% ethanol, air-dried, and eluted in 50 µL of TE buffer.
Qiagen DNEasy Plant Mini-kits were used to extract DNA from approximately 75 mg of fresh thallus or 10 mg of silica-dried thallus. Protocol was followed according to manufacturer’s instructions; genomic DNA was eluted with 60 µL of the provided AE buffer. For PCR amplification, DNA was diluted to 5-20% with sterile H2O.

4. PCR amplification:

DNA Sequence Analyses – A subset of Hawaiian A. spicifera samples was sequenced for each of these markers. Three DNA markers, one each from the nuclear, mitochondrial, and plastid genome, that have been successfully used to reveal population level variation were sequenced. All PCR reactions for sequencing were carried out in 50 uL volumes. The mitochondrial cox 2-3 spacer region was amplified and sequenced using previously published cycling conditions. A region of the nuclear ribosomal large subunit gene (LSU-Y) was also amplified and sequenced using the previously published cycling conditions. The chloroplast marker (partial 23S rRNA) was amplified using the following PCR conditions: 2 minutes at 94°C, followed by 20 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds.

All samples were sequenced according to the instructions of the University of Hawaii Greenwood DNA sequencing core facility. Additional samples of A. spicifera from throughout the Pacific Ocean (e.g. Guam, Okinawa, Australia) were sequenced.

Microsatellite loci – Seven microsatellite loci were amplified for 12 populations and approximately 450 individuals of A. spicifera in 10 µL reactions, a total of nearly 3,500 individual PCR reactions. Microsatellite PCR products were labeled with fluorescent markers that are compatible with the ABI sequencer at the CGPBRI facility. Reactions were run with the following cycling conditions: 2 minutes at 94°C, followed by 35 cycles of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds.

5. Multiplex Genotyping:

For microsatellite analysis, PCR products from all seven loci were pooled together and 1 µL of this mixture was submitted to the University of Hawaii CGPBRI core facility for genotyping.

F. Data analysis techniques:

1. Microsatellite raw data – The genotyping software, Genemarker, was used to convert the microsatellite raw data output (electropherogram) from the DNA sequencer. Results from electropherograms were sorted according to the size of each PCR product.

2. Sequence raw data - Bioedit software was used to examine and align the DNA sequence results.

3. Statistical analysis – Because no variation was detected in the sequencing component of this study, no statistical analyses were employed. Kinship software was employed to detect the occurrence of clonal genotypes in the microsatellite dataset. Genepop software was used to calculate several statistical measures of population structure.

G. Photos from research during each stage (see Appendix, Page 4)

H. Contact information for companies used to purchase items unique to your project

- Genetic Identification Services (microsatellite primer design)
  9552 Topanga Canyon Blvd.
  Chatsworth, CA 91311
  (818)718-9600

VI. Results
A. Findings for each IIIC objective

1 & 2. Results summary for genetic analyses –

- The sequencing component of the study revealed no variation for any of the three markers analyzed. Initially, we planned to sequence a subset of 60 individuals for each of the three sequence markers for a total of 180 sequences. However, a subset of 20 individuals selected from five distant Hawaiian populations failed to reveal any sequence variation. Since the introduction of *A. spicifera* to Hawaii likely represents a genetic bottleneck, we attempted to detect sequence variation in widely distributed samples from the species native range. We sequenced individuals from Guam, Australia, and Okinawa and again detected no variation in DNA sequence for any of these three markers. Although these markers have revealed population-level sequence variation in some seaweed species, universal markers that consistently provide intra-specific and population-level resolution within various taxonomic groups are not currently known. Nonetheless, it was unexpected to find no variation at the species level among widely ranging locations within *A. spicifera*’s natural distribution. Since no variation was observed, we chose to discontinue DNA sequencing of Hawaiian *A. spicifera*.

- In contrast, the microsatellite component of the analysis was extremely informative and demonstrated substantial population structure within 12 populations from Kaua’i, O’ahu, Maui, Moloka’i, and Hawai’i. Multi-locus genotyping using microsatellites is currently the highest resolution genetic marker system available. Genotyping provides a unique “genetic fingerprint” for each individual alga and was able to detect the occurrence of genetically identical samples, or clones, that are produced through vegetative fragmentation and re-growth.

  Genetic Identification Services (GIS) provided sets of PCR primers designed to amplify 24 different microsatellite loci. After preliminary testing of these markers, we optimized and selected seven loci for the final analyses. The remainder of the markers were discarded after an initial screening process or after high resolution genotyping revealed unscorable results: 14 markers were monomorphic or never produced PCR products, even after extensive optimization efforts, while three markers revealed null alleles, or un-interpretable electropherograms.

  The population genetics software, Kinship, detected many identical genotypes within each population sample. Most populations contained several different clonal genotypes with various numbers of samples in each genotype. The degree of clonal reproduction present in each population ranged widely. A population sample at Liliuokalani Gardens, Hilo, Hawai’i was composed of 34 clonal and two unique genotypes; in contrast, a population sample taken from shallow water adjacent to Ali’i fishpond, Moloka’i contained five different clonal genotypes, but only two or three individuals of each clone were detected, and the remaining 19 genotypes were unique to this locality. The wide range of variability in clonal reproduction among Hawaiian population of *A. spicifera* is summarized in Appendix, Page 2.

  The Genepop software was used to calculate descriptive statistics and apply a variety of statistical analyses appropriate for microsatellite markers. When considering all Hawaiian collections, the seven microsatellite loci revealed between three and six alleles per locus for a total of 27 alleles (Appendix, Page 2). Each population exhibited different degrees of genetic diversity, as measured by allelic diversity; no single population contained all 27 alleles and total alleles detected ranged from 12 alleles in populations in Kaua’i to 22 alleles from Iroquois Point, O’ahu (Appendix, Page 2). The population collected at the mouth of Pearl Harbor (Iroquois Point - the harbor is the purported location of initial introduction), contains the highest number of alleles among all collections. The island of O’ahu was the first Hawaiian island to be invaded by *A. spicifera* and still receives the highest intensity of boat traffic and anthropogenic activity.
Harbors are associated with hull-fouled ships, ballast water discharge, restricted water movement, and a plethora of artificial substrata (e.g. nets, ropes, floating docks), thereby creating a situation where new genets of *A. spicifera* are able to rapidly proliferate, sexually reproduce, and be transported to other locations. Thus, proximity to harbors and length of time since establishment is often associated with well-established populations with high genetic diversity. Not surprisingly, populations collected from O'ahu possess a higher number of alleles than other islands. In contrast, the islands of Hawaiʻi and Kauaʻi are genetically distant from the site of introduction, isolated from the Maui Nui complex and O'ahu by large channels and strong prevailing surface currents, and have fewer populations of *A. spicifera* than all other Hawaiian Islands. Not surprisingly, these islands appear relatively isolated and contain the fewest alleles. Several rare alleles were unique to O'ahu or Moloka'i were not found in collections from any other island. Also, one rare allele at each of two loci were shared between O'ahu and Moloka'i populations, suggesting occurrence of a small amount of genetic exchange between the two islands.

Quantification of alleles per population and unique alleles does not require adjustment of the dataset, but the unique sexual cycle of this species requires elimination of haploid individuals and clones before the application of statistical techniques. Microsatellites are widely used in zoological studies of natural populations, but clonality and haploidy are not common difficulties. Only five algal microsatellite studies have been published, and none of these were of red algae. The lack of statistical models appropriate for algal life histories and dispersal strategies, combined with differentially reduced population sample sizes, makes interpretation difficult.

Since occurrence of clonal reproduction was discovered to be prevalent in several populations, only one copy of each genotype was included in each analysis to prevent artificially biased statistical measures. Additionally, gametophyte individuals of *A. spicifera* contain haploid genomes (i.e. only one allele possible at each locus), and could not be included in statistical analyses that required calculation of heterozygosity. Calculation of allele frequencies, heterozygosity, and Fst estimates for each locus and population provided detailed estimates of genetic structure and revealed varying, but generally high degrees of differentiation among Hawaiian populations of *A. spicifera*. The high Fst estimates (Appendix, page 3) indicate that most of the genetic variation is found among populations (i.e. populations from different geographic locations are genetically distinct from one another). In addition to substantially different allele frequencies and Fst values, each population is characterized by the presence of several unique clonal genotypes.

3. Reproductive status results -

Sporophyte and gametophyte thalli of *A. spicifera* are identical unless they are actively producing spores or gametes. Male and female gametangial plants were only collected on Moloka'i and Kaua'i, but have been observed occasionally during informal collections on O'ahu. The number of tetrasporangia and or gametangia observed ranged widely across populations. Only two populations (Kamalo Wharf, Moloka'i and Liluokalani Gardens, Hawaiʻi) contained only sterile thalli; these two populations also have the highest number of clonal genotypes. The reproductive status of each population is summarized in Appendix, page 2.

B. Answers to IIB each resource management question

1: Populations of *A. spicifera* have changed in genetic structure as they dispersed through the Main Hawaiian Islands.

2: The substantial degree of variation revealed by microsatellite analyses suggests that introduction of this species was not limited to a single introduction event. It is more
likely that multiple or ongoing introductions are responsible for the highly structured patterns of genetic variation observed throughout the main Hawaiian islands.

3: Reproductive status appears to be associated with patterns in genetic diversity. Sterile populations would be expected to rely on fragmentation in order to establish the standing crop of algae found in the surrounding locality. In support of this, sterile populations are associated with high numbers of clones. For one population collection undergoing sexual reproduction (Ali‘i fishpond, Moloka‘i), we observed particularly high genotypic diversity. Although the O‘ahu population near Pearl Harbor (Iroquois Point) had the highest allelic diversity, no gametophytes were observed in this collection. In contrast, the population collected next to Ali‘i fishpond, Moloka‘i yielded several fertile male thalli, and one fertilized female thallus. Although allelic diversity was lower than at Iroquois Point, genotypic diversity was higher in the Moloka‘i population (i.e. the smaller number of alleles recombined via sexual reproduction to yield a greater number of genotypes).

C. Site specific results for each location (see Appendix, pages 1-3)

VII. Resource Management Implications

A. Given the results from VI, what are the implications for resource managers?

These results provide much-needed basic information for resource managers regarding the reproductive status and dispersal of invasive algae in the Hawaiian Islands. The results of this project will allow resource managers to make informed decisions concerning removal efforts or controlling the spread of this species based on patterns of genetic variation. The high degree of genetic structure observed in this macroalgal species suggests that native limu species may also be genetically structured, and could be evaluated with similar tools. The range of dispersal and connectivity between algal populations has numerous implications for the conservation of native species as well as control and prediction of the spread of invasive species.

B. How do these implications and results help to address the resource management problems identified in IIIA?

The results of this genetic and reproductive study answer a variety of questions regarding the most invasive alga in Hawaiian waters. Despite their widespread occurrence and increasing distribution, very little is known about population structure of invasive seaweeds in Hawai‘i or other tropical reef ecosystems. This project helps by addressing the reproductive strategies used by A. spicifera. The results suggest that the nuisance levels of biomass produced by this species are often maintained by vegetative fragmentation. We now have basic information concerning the degree of genetic structure, sexual reproduction, and dispersal, that will help resource managers target specific populations likely to respond favorably to control efforts.

C. What recommendations for resource managers can be made based on the implications and results?

Because the results of this study indicate that boats are a primary dispersal vector, it is recommended that regulations concerning hull fouling of all vessels be increased or enforced as much as possible to minimize the dispersal and recruitment of new populations. Recommendations based on the results of this study would concern the targeting of specific localities to test control efforts based on several criteria. The genetic
diversity, time elapsed since invasion, reproductive strategy, and degree of infestation, and are important considerations when selecting sites for control. Populations with low genetic diversity are more likely to lack the “genetic flexibility” to withstand control measures. Additionally, low genetic diversity may indicate the rarity of incoming propagules or a newly established population. If a new population can be removed quickly after introduction, the ecosystem balance at the locality would likely be less disrupted than well-established populations, and therefore be more apt to recover and resist recolonization; after removal, such a relatively isolated, low diversity population is not likely to be inundated with new propagules. Inter-tidal populations would be good targets for removal; they are easy to access at low tide and tend to produce more gametes and spores than sub-tidal populations. Currently, and in the future, these microsatellite markers can be used to identify possible sources of new populations and to identify potential candidates for targeting by resource managers. The field guide will be distributed to resource managers and provide instructions on collecting, identifying, and preserving samples of *A. spicifera* for subsequent analysis.

VIII. Evaluation

A. Objective #1

1. The genetic analyses objective was designed to determine the genetic structure of *A. spicifera*. The seven microsatellite loci used in the genotyping component of this project revealed a large amount of data that fully attained the objective. The microsatellite data were able to discern fine-scale genetic differences between individual samples and recognize the clonal structure of *A. spicifera*.

2. No modifications were made to Objective #1.

3. Because DNA sequencing yielded no genetic variation in a subset of Hawaiian samples or among samples from the Guam, Australia, and Okinawa, the sequencing of the remaining Hawaiian samples was discontinued.

4. No additional work is necessary to attain Objective #1. However, because microsatellite genotyping for the Main Hawaiian Islands collections was extremely informative, new collections can be made and novel hypotheses regarding dispersal and structure can be tested with the existing microsatellite markers.

**Objective #2**

1. Reproductive examination of field collections was used to evaluate the frequency, and possible seasonality of sexual reproduction.

2. No modifications were made to Objective #2.

3. No problems were encountered with Objective #2.

4. No additional work is necessary; we were able to determine the lack of seasonal reproduction from the collections for this project.

B. What performance measures are used to evaluate how well the project met the stated objectives?

As a measure of performance, the interpretation of microsatellite analysis and reproductive status were able to provide a numerous insights regarding clonal reproduction and dispersal, in addition to answering to the specific questions stated in IIIB. Removal efforts to be co-ordinated with DAR will be targeted to localities according to the results of this project; the degree of success attained for each algal removal experiments will be compared to randomly selected removal plots.

IX. Dissemination of project results:

A. Explain, in detail, how the projects results have been, and will be,
disseminated.

The project results have been disseminated for a number of audiences (e.g. laypeople, biologists, HCRI administrators, resource managers, and DAR and NOAA employees). The project will be expanded and refined to become a Master’s Thesis for the University of Hawai‘i Botany Department (Dan O’Doherty). This expanded genetic analysis of is one of the first algal microsatellite analyses to date and will likely be among the first microsatellite publications to investigate the population structure of an invasive red alga. Additionally, we are aiming to present this work at the Phycological Society of America conference this summer in Rhode Island.

B. List of publications, workshops, and presentations
1. HCRI Quarterly Meetings, Bishop Museum, 10-15 minutes each: January, May, September 2006, January 2007
2. Marine Progress Series, Hanauma Bay, 45 minutes plus questions: December 2006
3. CD-ROM virtual presentation, Hanauma Bay, approximately 10 minutes – These CDs contain a virtual powerpoint presentation that outlines the processes involved in typical genetic lab analyses. The presentation was designed to provide field collection collaborators (DAR partners), resource managers, and other interested parties with an understanding of the tools used and processes that take place in between field collections and interpretation of results. The powerpoint is automatically narrated and contains numerous images of laboratory equipment, as well as videos of field collection and laboratory procedures.

C. Data or information products
1. Field guide to identification and preservation of A. spicifera (Appendix, page 5)
2. Project website: easy to access information
   <http://www.botany.hawaii.edu/faculty/Sherwood/acanthophora/acanthophora.htm>

D. Partnerships established with agencies or organizations
   Partnerships were formed with DAR representatives and employees from:
   - Maui: Skippy Hau
   - Moloka‘i: Bill Puleloa
   - Island of Hawai‘i: Dr. Robert Nishimoto, Jennifer Vandeveur, Cecile Walsh
Site specific results

<table>
<thead>
<tr>
<th>Collection Location</th>
<th>Collection Date</th>
<th>Substrata</th>
<th>Depth</th>
<th>Morphological notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaalawai Beach, O‘ahu</td>
<td>January 23, 2006</td>
<td>reef flat with rubble, coarse sand</td>
<td>4-6 feet</td>
<td>plants small in stature and sparsely branched</td>
</tr>
<tr>
<td>Kualoa Beach Park, O‘ahu</td>
<td>February 3, 2006</td>
<td>lagoon and reef flat with rubble, coarse sand</td>
<td>3-4 feet</td>
<td>robust, well developed plants</td>
</tr>
<tr>
<td>Iroquois Point, O‘ahu</td>
<td>March 2, 2006</td>
<td>reef flat with rubble, coarse sand</td>
<td>3-6 feet</td>
<td>robust, well developed plants</td>
</tr>
<tr>
<td>Haleiwa Ali‘i Beach Park, O‘ahu</td>
<td>December 22, 2006</td>
<td>intertidal, low-tide collections from eroded lava</td>
<td>N/A</td>
<td>highly branched, short plants</td>
</tr>
<tr>
<td>Kahana, Maui</td>
<td>March 9, 2006</td>
<td>subtidal reef flat, rubble, coarse sand</td>
<td>6-10 feet</td>
<td>highly branched, extremely thin axes</td>
</tr>
<tr>
<td>Kanaha, Maui</td>
<td>March 10, 2006</td>
<td>subtidal reef flat, rubble, coarse sand</td>
<td>4-8 feet</td>
<td>robust, well developed plants</td>
</tr>
<tr>
<td>Ali‘i fishpond, Moloka‘i</td>
<td>April 21, 2006</td>
<td>shallow subtidal, mud flat with embedded rubble</td>
<td>1-3 feet</td>
<td>robust, well developed plants attached to rubble and lava buried in mud</td>
</tr>
<tr>
<td>Kamalo Wharf, Moloka‘i</td>
<td>April 21, 2006</td>
<td>subtidal reef flat, rubble, fine sediment</td>
<td>2-4 feet</td>
<td>moderately branched, small in stature</td>
</tr>
<tr>
<td>Liliuokalani Gardens, Hawaii‘i</td>
<td>May 31, 2006</td>
<td>subtidal, mud bottom</td>
<td>2 inches - 1 foot</td>
<td>extremely thin axes, plants laying unattached in mud</td>
</tr>
<tr>
<td>Hilo rocky tidepools, Hawaii‘i</td>
<td>May 31, 2006</td>
<td>wave-washed intertidal rocks and small tidepools</td>
<td>N/A</td>
<td>highly branched plants with thick axes, unusually high degree of branching near apices</td>
</tr>
<tr>
<td>Port Allen Harbor, Kaua‘i</td>
<td>August 20, 2006</td>
<td>wave-washed intertidal boulders</td>
<td>N/A</td>
<td>short, pale plants, many heavily bearing gametangia</td>
</tr>
<tr>
<td>Nawiliwili, Kaua‘i</td>
<td>August 20, 2006</td>
<td>intertidal, lava benches and boulders</td>
<td>N/A</td>
<td>short, pale plants, many with gametangia</td>
</tr>
</tbody>
</table>
## Appendix – Page 2

### Site specific results

<table>
<thead>
<tr>
<th>Collection Location</th>
<th>sterile</th>
<th>tetraporangial</th>
<th>Female</th>
<th>male</th>
<th># of alleles per population</th>
<th># different clonal genotypes</th>
<th>% of duplicated genotypes within population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaalawai Beach, O'ahu</td>
<td>38</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>19</td>
<td>9</td>
<td>58%</td>
</tr>
<tr>
<td>Kualoa Beach Park, O'ahu</td>
<td>21</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>4</td>
<td>21%</td>
</tr>
<tr>
<td>Iroquois Point, O'ahu</td>
<td>33</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>22</td>
<td>8</td>
<td>51%</td>
</tr>
<tr>
<td>Haleiwa Ali'i Beach Park, O'ahu</td>
<td>37</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>19</td>
<td>10</td>
<td>43%</td>
</tr>
<tr>
<td>Kahana, Maui</td>
<td>27</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>3</td>
<td>54%</td>
</tr>
<tr>
<td>Kanaha, Maui</td>
<td>15</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>19</td>
<td>6</td>
<td>66%</td>
</tr>
<tr>
<td>Ali'i fishpond, Moloka'i</td>
<td>18</td>
<td>17</td>
<td>1</td>
<td>4</td>
<td>18</td>
<td>5</td>
<td>20%</td>
</tr>
<tr>
<td>Kamalo Wharf, Moloka'i</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>4</td>
<td>78%</td>
</tr>
<tr>
<td>Liliuokalani Gardens, Hawai'i</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>1</td>
<td>94%</td>
</tr>
<tr>
<td>Hilo rocky tidepools, Hawai'i</td>
<td>20</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>10</td>
<td>60%</td>
</tr>
<tr>
<td>Port Allen Harbor, Kaua'i</td>
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<td>10</td>
<td>12</td>
<td>12</td>
<td>3</td>
<td>16%</td>
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### Appendix – Page 3

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1 – Diamond Head, Oahu  
2 – Kualoa, Oahu  
3 – Iroquois Point, Oahu  
4 – Kahana, Maui  
5 – Kanaha, Maui  
6 – Alii fishpond, Molokai  
7 – Kamalo Wharf, Molokai  
8 – Liliuokalani Gardens, Hawaii  
9 - Hilo rocky intertidal, Hawaii  
10 – Haleiwa, Oahu  
11 – Port Allen, Kauai  
12 – Nawiliwili, Kauai
Images from top to bottom and left to right:
1. Live *Acanthophora spicifera* plant, 2. image of collection site, 3. lab tools for DNA extraction, 4. field collection of *A. spicifera*, 5. stereomicroscope, 6. microscope image of tetrasporangia, 7. agarose gel electrophoresis showing different allele sizes
1. Collecting

Preserving seaweeds for molecular analyses

Siliq gel protocol for collecting and preserving Acanthophora spicifera

2. Preserving

Sample in a generous amount of seawater.

Possibly of acquiring the same plant species. Store pairs the very plant, if possible. If not, immediately add each sample to a cold, 4°C suspension of seawater or preservation medium. Include the sample in the prepared stock suspension.

A suspension of seawater or 4°C suspension of seawater is recommended. For both samples, it is added to a well-mixed, sterile seawater suspension. The number of seawater samples is typically at least 2, depending.

In conclusion, seawater samples may support dark, shore microscopic and aquatic life more naturally. Suitable conditions for longer-term preservation include cold temperatures, 4°C.