APPENDIX ES-5: OAHU ARMY NATURAL RESOURCE PROGRAM RESEARCH PROPOSAL, M. EUAPARADORN

Oahu Army Natural Resource Program Research Proposal

Title: Pollination biology of *Euphorbia celastroides* var. *kaenana* (Euphorbiaceae).

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Type of Support Requested: Research Assistantship

Proposed Project Period: June 2010 – May 2011

Statement of Problem:

Pollinators and pollination can affect the viability of plant populations, especially those of rare plants. Although the disruption of pollination systems and loss of pollinators have been attributed to the decline of several endangered species in Hawaii, little remains known of the pollination biology of most Hawaiian plants. For the successful recovery of these endangered plant species, basic information on their breeding system must be determined and incorporated into management programs.

Plant pollination systems of rare plants may be especially sensitive to the effects of habitat fragmentation. Reduction of habitat can reduce plant and pollinator relative abundance and pollinator species richness, resulting in reduced pollination services. These reduced plant populations are more likely to suffer from inbreeding depression as self-fertilization occurs from pollinators visiting a higher proportion of flowers on individual plants.

The endemic Hawaiian plant *Euphorbia celastroides* var. *kaenana* (Euphorbiaceae) is known only from fragmented populations on the island of Oahu on the northwestern end of the Waianae Mountains and a single collected specimen from the southeastern portion of the Koolau Mountains. With the decline of populations of *E. celastroides* var. *kaenana*, the species was listed as endangered in 1991. The major objective of this study is to investigate the pollination and reproductive biology of *E. celastroides* var. *kaenana,* as little is known of its breeding system, to provide vital information for the recovery of this species.

Procedures/Methods:

Study Sites - It is proposed that this study be conducted at populations of *E. celastroides* var. *kaenana* occurring within Kaena Point NAR, Kaena Point State Park, and Makua Military Reservation. The Kaena Point NAR and the Makua Military Reservation populations will be

categorized as larger, contiguous populations, while the populations at Kaena Point State Park will be considered smaller, isolated populations (Table 1).

Table 1. Proposed populations units of *E. celastroides* var. *kaenana* to be included in study.

Land Ownership	Population Unit	Total No. of Individuals	Population Size Category
Kaena Point NAR	Kaena Point	375-525	Larger, Continuous
Kaena Point State Park	East of Alau	26	Smaller, Isolated
Kaena Point State Park	Kaewaulu (population C & D)	20	Smaller, Isolated
Makua Military Reservation	Lower Ohikilolo	118	Larger, Continuous

Note on floral terminology – The flower of *E. celastroides* var. *kaenana* is known as a cyathium. Each cyathium is an inflorescence of several male (staminate) flowers and one central female (pistillate) flower.

Selection and manipulation of plants – When randomly selecting plants to be manipulated, individuals with a low number of flowers will be excluded. For each individual that is selected, manipulation of flowers will be limited to less than 20% of the flowers produced in the season.

Objective 1: Determine the phenology of *E. celastroides* var. *kaenana* by measuring cyathia and fruit production monthly.

- *Purpose* – A basic understanding of phenology will be pertinent for future research and management of *E. celastroides* var. *kaenana.*

- *Methods* – Total number of buds, cyathia, and fruit will be counted on ten randomly selected individual plants monthly.

Objective 2: Determine if *E. celastroides* var. *kaenana* is self-compatible or an obligate out-crosser.

- *Purpose* – If *E. celastroides* var. *kaenana* is found to be self-incompatible and requires cross-pollination for seed production, it will stress the importance of managing for both the protection of the pollinators and a genetically diverse population of *E. celastroides* var. *kaenana.*

In the outcross treatments, seed set will also be compared between cyathia cross-pollinated with a near versus a far plant donor. In general, when pollen and seeds are dispersed a short distance, neighboring plants are generally more closely related to each other than distantly separated plants. As a result, these closely related individuals are more likely to cross-pollinate leading to inbreeding depression.

- *Methods* – One hundred and twenty randomly selected buds from ten flowering individual plants will be bagged with a fine mesh and randomly assigned to one of four treatments: (1) no manipulation (autogamy) – no hand pollination of cyathium, (2) induced self-pollination (geitonogamy) – cyathium hand pollinated with pollen of the same plant donor, (3) near donor cross-pollination (xenogamy) – cyathium hand pollinated with a mixed pollen load from neighboring plant donors, and (4) far donor cross-pollination (xenogamy) – cyathium hand pollinated with a mixed pollen load from distant plant donors.

  Cyathia in Treatment (3) and (4) will be emasculated before stigmas are receptive to avoid self-fertilization. Pollen from these emasculated anthers will be used to pollinate other cyathia upon anthesis. After approximately 1 month, fruits will be collected to compare total number of fruit and seeds set between treatments.

Objective 3: Determine if seed set in *E. celastroides* var. *kaenana* is pollen limited.

- *Purpose* – This will determine whether reproductive success in *E. celastroides* var. *kaenana* is limited by insufficient deposition of pollen on stigmas.

- *Methods* – Sixty randomly selected buds from ten flowering individual plants will be randomly assigned to one of two treatments: (1) open pollination - cyathium left uncovered and no experimental manipulation, and (2) cross pollination (xenogamy) - cyathium bagged and hand pollinated with pollen from a different plant (these will be the same flowers from Treatment 4 of Objective 2). After approximately 1 month, fruits will be collected to compare total number of fruit and seeds between treatments.

Objective 4: Determine if seed set in *E. celastroides* var. *kaenana* is limited by pollen viability and/or stigma receptivity.

- *Purpose* – Reproduction in rare plant species may be limited by male and female infertility. Determining pollen viability and timing of stigma receptivity are also imperative for flower manipulation studies.

- *Methods* - A chemical test will be used to estimate the viability of pollen and receptivity of stigmas. To determine pollen viability, pollen will be collected from freshly dehisced anthers from 10 cyathia and stained with a chemical that tests for

dehydrogenases as an indication of viability. Ten stigmas from 1-day, 2-day and 3-day old cyathia will be similarly tested to determine the developmental stage at which stigmas are most receptive.

Objective 5: Identify floral visitors and determine their ability to effect pollination in *E. celastroides* var. *kaenana* by quantifying visitation rates and pollen carrying load.

- *Purpose* – Identifying the floral visitor community is of general interest and important for future research and management of *E. celastroides* var. *kaenana.* To determine the effectiveness of these floral visitors to effect pollination, rates of visitation and pollen carrying load will be examined.

- *Methods* - Composition of the floral visitor community and rates of visitation will be quantified by observing insect activity at adjacent cyathia during 10 minute periods between 0900 and 1500. Cyathia will be observed from a 1-meter distance using close focusing binoculars. For each floral visitor, the identity of the visitor, whether or not it contacted the cyathium's reproductive parts, the floral resource collected, and the duration of the visit will be recorded. Observations will be conducted every two weeks during peak flowering periods on dry days with sunny weather and moderate wind speeds.

  To examine the extent to which various taxon are capable of transporting *E. celastroides* var. *kaenana* pollen, insects observed foraging on cyathia will be collected and examined for presence of pollen. A representative of 10 individuals from each taxon will be collected.

  A reference collection of the floral visitors of *E. celastroides* var. *kaenana* will be compiled and housed at the Bishop Museum. Species level identifications will be made using keys and museum collections.

Objective 6: Determine if smaller, isolated populations of *E. celastroides* var. *kaenana* receive fewer visits by floral visitors compared to the larger, more contiguous populations.

- *Purpose* – In plants that rely on insects as pollinators, small plant populations in fragmented habitats will tend to have lower seed set due to a reduction in pollinator abundance and richness. For future management and conservation of *E. celastroides* var. *kaenana*, it is important to determine if floral visitation rate and seed set is reduced in smaller, isolated populations compared to larger, continuous populations.

- *Methods* - Rates of visitation by floral visitors (Objective 5) will be compared between the smaller and larger populations of *E. celastroides* var. *kaenana.*

Objective 7: Determine if seed set and seed viability in *E. celastroides* var. *kaenana* reduces with population size as an indicator of the effects of inbreeding depression.

- *Purpose* – Studies have shown that plant populations become inbred at greater rates in smaller than larger populations. A typical symptom of inbreeding depression is a reduction in seed set and seed viability.

- *Methods* - Seed set and seed viability (Objective 3, Treatment 1) will be compared between plants of the smaller and larger populations of *E. celastroides* var. *kaenana*. Seed mass and chemical testing with Tetrazolium chloride (TTC) will be used to assess seed viability.

Objective 8: Determine if experimentally reducing inbreeding levels in both small and large populations affects seed set and seed viability in *E. celastroides* var. *kaenana*.

- *Purpose* – Seed set and viability have been shown to be reduced in smaller populations, because of reduction in pollinator visitation rates, an increase in the likelihood of crossing with close relatives, and a lack of vigor of seeds produced. If seed set is increased in the smaller populations when cyathia are cross-pollinated with cyathia of the larger populations, it will provide further support that seed set in the smaller population is affected by population size.

- *Methods* - Seed set and seed viability will be compared between cyathia hand pollinated with pollen from the same population and cyathia hand pollinated with pollen from a different population in both small and large populations. Hand pollination trials will be conducted in a greenhouse with propagated plants originating from both the small and large populations. Seed mass and chemical testing with Tetrazolium chloride (TTC) will be used to assess seed viability.

Objective 9: Identify whether ant floral visitation reduces seed set and seed viability in *E. celastroides* var. *kaenana* at the Kaena population unit.

- *Purpose* – From preliminary floral observations, ants have been observed to be the dominant floral visitor of *E. celastroides* var. *kaenana*. In general, ants are regarded as poor pollinators, because pollen does not readily adhere to their bodies and antibiotics secreted by ants to combat fungal growth reduces the viability of pollen. Ants may also limit seed set and viability in plant populations by both diminishing the amount of available nectar and aggressively deterring pollinators at flowers.

- *Methods* - Seed set and seed viability will be compared between the following cyathia manipulations: 1) cyathia excluded from ants allowing access to only flying insects; 2) cyathia excluded from flying insects allowing access to only ants; 3) cyathia excluded from both ants and flying insects; 4) cyathia open to all visitation.

To exclude only ants from a cyathium, Vaseline will be applied to the base of a branch to function as a trapping adhesive. To exclude flying insects while still allowing access by ants, mosquito netting will be draped over a cyathium and secured. An entire cyathium will be bagged to eliminate both ants and flying insects. Seed mass and chemical testing with Tetrazolium chloride (TTC) will be used to assess seed viability. Thirty flowers will be used in each treatment from 10 different individual plants.

Table 2. Research objectives to be conducted per Population Unit of *E. celastroides* var. *kaenana* from June 1, 2010 through May 31, 2011.

		Population Unit			
Obj. #	**Methods**	**Kaena Point**	**East of Alau**	**Keawaulu**	**Lower Ohikilolo**
1	Monitor phenology. Count total number of buds, cyathia, and fruit monthly.	✓			
2	Test for self-compatibility. Four treatments: (1) no manipulation, (2) induced self-pollination – cyathium hand pollinated with pollen of the same plant, (3) near donor cross pollination – cyathium hand pollinated with pollen from a near donor, and (4) far donor cross pollination – cyathium hand pollinated with pollen from a far donor.	✓			
3	Test for pollen limitation. Two treatments: (1) open pollination - cyathium left uncovered and no experimental manipulation, and (2) cross pollination - cyathium bagged and hand pollinated with pollen from a different plant (*these will be the same flowers from Treatment 4 of Objective 2*).	✓	✓	✓	✓
4	Chemical test of pollen viability and stigma receptivity. Pollen from freshly dehisced anthers, and 1-, 2-, and 3-day old stigmas will be tested.	✓			
5	Quantify insect visitation rate and pollen carrying load. Visits by insects will be recorded in 10 min. intervals. Thirty individuals of each visitor taxon will be collected and examined for presence of pollen.	✓	✓	✓	✓

Table 2 (*continued*). Research objectives to be conducted per Population Unit of *E. celastroides* var. *kaenana* from June 1, 2010 through May 31, 2011.

Obj. #	Methods	Population Unit: Kaena Point	East of Alau	Keawaulu	Lower Ohikilolo
6	Test if plant population size affects visitation rates. Rates of visitation by floral visitors (Objective 5) will be compared between the smaller and larger plant populations.	✓	✓	✓	✓
7	Test for the presence of inbreeding depression. Seed set and seed viability will be compared between the smaller and larger plant populations. *These will be the same flowers and fruit from Treatment 1 of Objective 3*.	✓	✓	✓	✓
8	Experimentally reduce inbreeding levels. Seed set and seed viability will be compared between cyathia hand pollinated with pollen from the same population and cyathia hand pollinated with pollen from a different population in both small and large populations. *To be conducted in the greenhouse with propagated plants.*				
9	Test whether ant visitation reduces seed set and seed viability. Seed set and seed viability will be compared between: 1) cyathia excluded from ants allowing access to only flying insects; 2) cyathia excluded from flying insects allowing access to only ants; 3) cyathia excluded from both ants and flying insects; 4) cyathia open to all visitation.	✓			

Table 3. Summation of total number cyathia to be manipulated and fruits to be collected of *E. celastroides* var. *kaenana* per research objective from June 1, 2010 through May 31, 2011.

Obj. #	Methods	# of plants	Total # of cyathia	Removal and/or damage of cyathia	Collect fruits*
1	Monitor phenology. Count total number of buds, cyathia, and fruit monthly.	10	n/a	no	no
2	Test for self-compatibility. Four treatments: (1) no manipulation, (2) induced self-pollination – cyathium hand pollinated with pollen of the same plant, (3) near donor cross pollination – cyathium hand pollinated with pollen from a near donor, and (4) far donor cross pollination – cyathium hand pollinated with pollen from a far donor.	10	120 (30/ treatment)	no	yes
3	Test for pollen limitation. Two treatments: (1) open pollination - cyathium left uncovered and no experimental manipulation, and (2) cross pollination - cyathium bagged and hand pollinated with pollen from a different plant (*these will be the same flowers from Treatment 4 of Objective 2*).	10	60 (30/ treatment)	no	yes
4	Chemical test of pollen viability and stigma receptivity. Pollen from freshly dehisced anthers, and 1-, 2-, and 3-day old stigmas will be tested.	10	40	yes	no
5	Quantify insect visitation rate and pollen carrying load. Visits by insects will be recorded in 10 min. intervals. Thirty individuals of each visitor taxon will be collected and examined for presence of pollen.	10	n/a	no	no

* Any seeds not tested with TTC will be properly stored at the OANRP facilities.

Table 3 (*continued*). Sum Summation of total number cyathia to be manipulated and fruits to be collected of *E. celastroides* var. *kaenana* per research objective from June 1, 2010 through May 31, 2011.

Obj. #	Methods	# of plants	Total # of flowers	Removal and/or damage of flowers	Collect fruit*
6	Test if plant population size affects visitation rates. Rates of visitation by floral visitors (Objective 5) will be compared between the smaller and larger plant populations.	10	n/a	no	no
7	Test for the presence of inbreeding depression. Seed set and seed viability will be compared between the smaller and larger plant populations. *These will be the same flowers and fruit from Treatment 1 of Objective 3*.	*same as Objective. 3, Treatment 1*	*same as Objective. 3, Treatment 1*	no	yes
8	Experimentally reduce inbreeding levels. Seed set and seed viability will be compared between cyathia hand pollinated with pollen from the same population and cyathia hand pollinated with pollen from a different population in both small and large populations. *To be conducted in the greenhouse with propagated plants.*	n/a	n/a	n/a	n/a
9	Test whether ant visitation reduces seed set and seed viability. Seed set and seed viability will be compared between: 1) cyathia excluded from ants allowing access to only flying insects; 2) cyathia excluded from flying insects allowing access to only ants; 3) cyathia excluded from both ants and flying insects; 4) cyathia open to all visitation.	10	120 (30/ treatment)	no	yes

* Any seeds not tested with TTC will be properly stored at the OANRP facilities.

Table 4. Total number of cyathia to be manipulated and fruits to be collected per Population Unit of *E. celastroides* var. *kaenana* from June 1, 2010 through May 31, 2011.

Population Unit	Total # of cyathia to be manipulated	Total # of fruit to be collected
Kaena Point	340	300
East of Alau	60	60
Keawaulu	60	60
Lower Ohikilolo	60	60
Grand Total	**520**	**480**

Anticipated Results and Products:

Results from this research will provide a valuable understanding of the pollination and reproductive biology of *E. celastroides* var. *kaenana*. Identification of potentially reproductive and ecological limiting factors will help facilitate the design of management strategies that will contribute to the recovery of this species.

A reference collection of the floral visitors of *E. celastroides* var. *kaenana* will be compiled and housed at the Bishop Museum. Species level identifications will be made using keys and museum collections.

A summary of results will be made available through a technical report, at least one peer reviewed journal, and a presentation at the Hawaii Conservation Conference.

Semester Timeline:

Summer 2010 (June-August 2010) – Monitor phenology, conduct flower visitor observations, hand pollination experiments, and floral visitor exclusion experiments.

Fall 2010 (September–December 2010) – Monitor phenology, conduct flower visitor observations, hand pollination experiments, floral visitor exclusion experiments, collect and test seeds, and insect pollen washing.