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8:30 A.M. - 4 P.M.

Hosted by

New Jersey City University

Jersey City, NJ

Conference Theme

Mosquitos as Vectors: Zika and Malaria

Instructions for Authors

IN VIVO is published three times yearly during the Fall, Winter, and Spring. Original research articles in the field of biology in addition to original articles of general interest to faculty and students may be submitted to the editor to be considered for publication. Manuscripts can be in the form of a) full length manuscripts, b) mini-reviews or c) short communications of particularly significant and timely information. Manuscripts will be evaluated by two reviewers.

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In This Issue:

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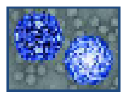
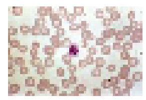
50th Annual Fall MACUB Conference

Saturday, October 28, 2017, 8:30 A.M.-4 P.M.

New Jersey City University, Jersey City, NJ

Conference theme with speakers below:

Mosquitos as Vectors → Zika and Malaria

Vincent Racaniello, Ph.D.
Higgins Professor, Department of Microbiology and Immunology
Columbia University College of Physicians and Surgeons

Vincent Racaniello today (left) and as a Ph.D. student in the laboratory of Peter Palese, Mt.

Dr. Racaniello studies the infection of humans with viruses ranging from Zika to the common cold. Dr. Racaniello completed both his undergraduate and his Ph.D. studies at Cornell University where he studied genetic reassortment of influenza virus. As a post-doctoral fellow in David Baltimore's laboratory at MIT (1979–1982), Racaniello used recombinant DNA technology to clone and sequence the genome of the small RNA animal virus poliovirus. He produced the first infectious clone of an animal RNA virus, which helped to greatly advance the field of modern virology.

With the global decline of poliovirus, Racaniello's lab has taken a particular interest in Zika virus. Racaniello's virology blog and podcasts *This Week in Virology*, help scientists and non-scientists alike learn more about viruses. He is a co-author of *Principles of Virology*, a textbook used by many students.

Ana Rodriguez, Ph.D.
Associate Professor, Department of Microbiology
Co-Director, Anopheles Insectary
New York University Medical Center

Cage of selected female Anopheles mosquitoes

Dr. Rodriguez's lab studies two different parasites, Plasmodium, which causes Malaria, and *Trypanosoma cruzi*, which causes Chagas disease. Malaria is a devastating disease that causes about 400,000 deaths per year, mainly among children in Africa. There is an urgent need for new strategies to control malaria, but there is a lack of detailed knowledge of the basic biological processes of Plasmodium, that would allow faster development of anti-malaria drugs and vaccines. A main interest of her laboratory is the study of malaria-induced inflammatory pathology and its implications in the pathology of disease, including cerebral malaria and severe anemia. The laboratory is attempting to develop effective drugs against Chagas Disease. In collaboration with GSK, her lab team has performed high through-put screenings of intracellular *Trypanosoma cruzi*, to find compounds with anti-trypanosomal activities. Selected compounds are now being tested for efficacy in mice.

Vouchered Plant Lists—Do We Need Them

Richard Stalter

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Abstract

The purpose of this paper is to present evidence for the need to base published floristic and plant ecological papers on vouchered vascular plant species. Where there is no vouchered plant material it may be impossible to verify the occurrence of taxa that may have been misidentified. Vouchered vascular plant specimens should have the minimum information on an herbarium label: genus, species, family, collector, asssession number, site, habitat and date of collection. Vouchered plant specimens will enable future investigators to record changes in species composition at a site over time. Rare taxa need to be verified and placed on maps for site location and verification in the future. Global positioning information to pin point the location of rare taxa is helpful but should only be retained by the collector, herbaria where the specimens are housed and site ie, city, state, National Parks, and National Wildlife Refuges where the taxa were collected. GPS information should only be distributed to reliable researchers to protect small populations of plants and rare taxa from unscrupulous collectors and from extirpation.

The objective of this paper is to present evidence for the need to base published floristic and plant ecological papers on vouchered vascular plant species. The need for basing published floristic studies based on vouchers is elegantly stated by Dr. Steve Young. Dr. Steve Young, Chief Botanist, New York Natural Heritage Program, states, "Well, for me, Richard, the biggest problem is trying to track down rare species listed in unvouchered plant lists. Instead of having a specimen that we can confirm, enter into the database, and put on the maps for protection, we have to treat the species on the list as a lead and take time to search for it to confirm it. Even the best botanists make mistakes and without vouchers flora lists are often looked at skeptically, especially if they are published by young botanists who have not built up a reputation. Lists in journal publications should always have vouchered specimens to back them up."

There have been several recent publications dealing with floras at northeastern United States based on incompletely vouchered and/or misidentified vascular plant species at urban parks[1] and at Inwood Park, New York[2]. Loeb[1] compared the vascular plant species at ten northeastern urban parks, and concluded that, "a common urban park flora does not exist." Only two of the ten floras in Loeb's paper were based on complete vouchers, Pelham Bay Park, New York[3] and Rock Creek Park, Washington D.C.[4]. Three floras, Middlesex Fells, Boston, Massachusetts[5] Van Cortland Park, New York City, New York[6] and Oregon Ridge, Baltimore, Maryland[7] were unvouchered[8]. Five studies were incompletely vouchered including three "in house" studies by Venezia and Cook[9] at Breezy Point, Jamaica Bay Wildlife Refuge, all units within Gateway National Recreation Area, New York, the United States' largest urban park. Venezia and Cook[9] listed 225 vascular plant species at Breezy Point of which 25, 11% of the total, were vouchered. Three hundred and seventy vascular plant species of their second incompletely vouchered inhouse list were reported at the Jamaica Bay Wildlife Refuge, while a completely vouchered list of taxa at Jamaica Bay Wildlife Refuge by Stalter and Lamont[10] containing 456 species, was omitted from Loeb's[1] study.

In a second study of the historical ecology of Inwood Park, New York, Fitzgerald and Loeb[2] reported, "The salt marsh community sample contained 64 species from 58 genera and encompassing 27 families." Their paper, Table 5, p. 291 listed species present in more than half of the salt marsh quadrats: *Amorpha fruticosa Calystegia sepium*, *Carex vulpinoidea*, *Celastrus orbiculatus*, *Dactylis glomerata*, *Plantago major*, *Rumex crispus*, *Taraxacum officinale*, *Trifolium pratense* and *Trifolium repens* (Table 1). Fitzgerald and Loeb[2] cite an earlier paper by Loeb[1] who reported the sand dune species, *Ammophila breviligulata*, in the Inwood Park salt marsh. None of the above species occur in salt marshes where tidal flooding and water and soil salinity control the distribution of vascular plant species found there. Fitzgerald and Loeb[2] did not collect vouchers because they were not allowed to collect plant material in Inwood Park. That they were not allowed to collect plant material is another egregious example of poor policies in city state and National Parks.

Stalter had a similar collecting experience in a study at Garden Key, Dry Tortugas National Monument, Florida. Stalter was permitted to collect vascular plant vouchers on his first foray, but not on subsequent trips to Garden Key. Further, Everglades National Park's policy mandated that all plant collections be subjected to -70°C treatment before the plant material left the park. While freezing plant material at -70°C is standard procedure for exchanging dry vascular plant vouchers, it is not appropriate for "uncured" taxa collected in the field. Stalter advised park personnel that while freezing plants is appropriate when herbarium material is exchanged, freezing collected plant material in a plant press would render some specimens unfit for voucher material. Stalter's advice was not heeded; vascular plants in his press were frozen and a number of the plants were destroyed.

The need for vouchered lists is apparent when considering the salt marsh species listed in Inwood Park by Fitzgerald and Loeb[2]. It is doubtful that their aforementioned species were misidentified as they are excellent botanists. Vouchered plant material in their study would have enabled future researchers to verify the habitat/location of the salt marsh species listed by them. In a second questionable habitat location, Loeb[1] reported the dune species, *Ammophila breviligulata*, was collected in the Inwood Park salt marsh.

Stalter[11] studied a Georgetown, South Carolina salt marsh as a dissertation research project which he completed in 1968. Since 1968, Stalter has conducted numerous floristic and ecological studies including the following: Biscayne National

Table 1. Density per ha of species present in more than half of the salt marsh quadrats, Inwood Park, New York. Data From Fitzgerald and Loeb[2].	
Species	Density
Amorpha fruticosa	1250
Calystegia sepium	2750
Carex vulpinoidea	1750
Celastrus orbiculatus	3500
Dactylis glomerata	7750
Plantago major	4750
Rumex crispus	5750
Taraxacum officinale	5250
Trifolium pratense	8000
Trifolium repens	5000

Park, Florida[12], north central coastal Florida[13], Turtle Island, South Carolina[14], Hunting Island, South Carolina[15], Bull Island, South Carolina[16], the authors' dissertation work at a South Carolina salt marsh, Georgetown, South Carolina[11], transplantation of salt marsh vegetation, Georgetown, South Carolina[17], The Outer Banks of North Carolina[18], Fisherman Island, Virginia[20], Assateague Island, Virginia[19], Cape May Point State Park, New Jersey[21], Sandy Hook, New Jersey[22], Jamaica Bay Wildlife Refuge, New York[23], Fire Island, New York[24], Monomoy Islands, Massachusetts[25]. Additional papers by Stalter in coastal environments include: "Observations on coastal plant communities of New York and New Jersey"[26] "Maritime communities in southeastern United States"[27] and The effect of superstorm Sandy on the vascular flora of the New York Bight[28]. In none of the aforementioned papers were the taxa cited by Loeb[1] and Fitzgerald and Loeb[2] observed in east coast salt marsh communities.

The need for vouchered plant species material is also apparent when native *Celastrus scandens* was reported by Venezia and Cook[9] at Breezy Point, New York, while the ubiquitous invasive *C. orbiculatus* was absent from their list. Additional non-existent taxa on the Venezia and Cook[9] list, cited by Loeb[8], allegedly occurring at Breezy Point, were *Viburnum acerifolium*, *Asimina triloba*, *Morus rubra*, and *Platanus occidentalis*. Missing from the Venezia and Cook[9] list were the exotic *Platanus acerifolium* and *Morus alba*. Michael Byer Ph.D., National Park Service Botanist Gateway National Recreation area, has not observed any of the aforementioned Venezia and Cook[9] vascular plant species at Breezy Point during the 20 years he has worked there.

Stalter[14] is guilty of using inferior herbarium paper to voucher plant specimens collected at Turtle Island, South Carolina. Plants mounted on inferior herbarium paper are worthless as voucher material.

Where there is no vouchered plant material, it may be impossible to verify the occurrence of taxa that may have been misidentified. Scientists might visit the site in the future to search for the missing vascular plant species, but if years have passed, one might argue that these taxa did occur at the site in the past and have disappeared over time.

Vouchered vascular plant specimens should have the minimum information on a herbarium label (Figure 1). Most herbarium labels have the following information: genus, species, family, collector, date of collection, site, habitat, and assession number. Assession number and herbarium where the plant specimen is housed enables the investigator to access these specimens on line. Labels on rare plants might also include GPS coordinates, though the author believes that this information might be best kept by the author and herbarium where the voucher is housed. Listing coordinates on a herbarium sheet would enable unscrupulous collectors to locate and wipe out a population of rare plants such as orchids. GPS information should also be housed with the agency where the specimens were collected such as city, state, and National Parks, and Fish and Wildlife Refuges. Coordinates would enable legitimate researchers to locate these rare taxa for comparative work in the future.

Figure 1. Herbarium label with pertinent information. Assession number is located in the upper right

Genus: Hibiscus 20434
Species: moscheutos
Family: Malvaceae
Habitat: Marshes, freq.
Locality: Caledon State Park, VA.
Collector: R. Stalter
Date: July 22, 2015

Vouchered plant specimens enable future investigators to record changes in species composition at a site over time. Changes in species composition may be a result of natural factors and forces e.g. community development (plant succession) that alters environmental conditions at a site that may render the habitat unfit for the survival of early light-loving succession species. Anthropomorphic disturbance such as mowing, brush-hegging, and burning also alter the environment often extirpating certain species. Natural events such as lightning caused fires, wind-throw (during hurricanes and nor'easters) may also cause the demise of certain vascular plant species. Thus, we conclude that there is a need for vouchered vascular plant species when preparing flora lists.

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Co-evolution and Adaptations in Milkweed Family -aligning ancient and modern tools to promote botany

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Abstract

Plants struggle in a variety of ways to survive under environmental stress. These modifications may be morphological, embryological, anatomical or physiological. Milkweed family has many members that have evolved to survive extreme conditions in the tropics by modifying the way plants get pollinated. Orchidaceae has some members with pollen aggregates known as pollinia. With re-categorization of some plants from Asclepiadaceae to Apocynaceae, pollinia are reported in Apocynaceae too. Pretty orange-flowered milkweed in the United States is known for its co-evolution with the monarch butterfly. An Indian Milkweed, *Calotropis procera,* found in arid conditions in tropics, is a short shrub with phytotoxic leaves, lavender flowers, and milky thick white latex. Rare visit by pollinators has caused *C. procera* and some members of Asclepiadaceae (milkweed family) to have pollen packaged in waxy pollen sacs known as pollinia or pollinaria. These pollen sacs are attached to a complex carrier known as translator apparatus. Instead of forming just the exine for each pollen grain, the plant develops a complex translator apparatus to be picked by the pollinator. Detailed insight on the ontogeny and histochemistry of the translator apparatus and importance of organismal botany for DNA barcoding will be discussed. Re-categorization of Asclepiadaceae into Apocynaceae has also been explored using FASTA files of some representative members. This article includes phylogenetic trees constructed using SmartBlast and DNA subway to show relationships among Apocynaceae,

Introduction

As a discipline, botany is surviving mainly as field botany, plant physiology, and recently as ethnobotany. Connecting embryology and anatomy with taxonomy is a rare exercise at the present time. Although knowledge of the diversity and commonality among vegetative and reproductive internal features can lead to construction of meaningful phylogenetic trees using Bioinformatics. Plant behavior or tropism in response to different environmental factors is studied at a very superficial level. Some misconceptions of tropisms are creating deceptive arguments about the presence of nervous system in Plants[1]. Plant hormones that play important role in growth, development and flowering are not always specific in their mode of action and may behave differently under different environmental conditions. There are very few generalizations that are known for plants in relation to flower structures, pollinators, chemistry of seeds, and role of hormones to name some. Even the types of embryo sacs and structures within the embryo sac can vary from one family to another. The nutritive tissue, endosperm, formed by double fertilization in angiosperms is not found in Orchidaceae because of difference in the number of cells in the embryo sac. Plant morphology, anatomy, and embryology with Latin terminology have been out of curriculum for too long in secondary education. Although, plant systematics has evolved recently based on DNA science and now with barcoding available, students will not have to worry about the identification of plants. Rather than understanding orders and families, students will be able to access the name and family of a plant based on its DNA barcode. However, with a basic understanding of morphological features, students can better use DNA Subway to construct rooted phylogenetic trees to be able to draw meaningful conclusions of evolutionary pathways describing the adaptations and survival of plants in different parts of the world. A simple structure like pollinium has no mention in biology or even introductory botany books.

Importance of Plant Morphology and DNA Science in Taxonomy

Asclepiadaceae (Milkweed family) and Orchidaceae were the only families that were known to have aggregates of pollen as pollinia or pollinaria. Recently, Apocyanaceae is involved in re-categorization of plants from Asclepiadaceae and thus have some members with pollinia. These unique aggregates of pollen may be enclosed within a thick sac or might contain a complex carrier known as Translator Apparatus. My Master's Dissertation some four decades ago was to study the origin and histochemistry of the Translator Apparatus in *Calotropis procera*. With some details that are known for these structures, DNA science can help us to better analyze divergence of families and learn why some plants store their pollen in sacs known as pollinium rather than yellow dust to be carried by the pollinators. Knowledge of diversity in male or female reproductive structures can surely help to resolve phylogenetic discrepancies and provide an understanding of ecological adaptations for the species. This article will highlight some details of embryological details about the survival of a plant from India, a type of milkweed. My intent is to inspire those with resources and knowledge to explore the internal features of plants as each plant has a story of survival to share.

Milkweed and its relationship with the monarch butterfly in the United States make a successful story of co-evolution. While observing milkweed flowers on Long Island, I am always reminded of *Calotropis procera,* called rubber bush, Indian milkweed, apple of Sodom, aak or rooster tree by USDA, which is only reported in California, Puerto Rico and Hawaii in North America (http://plants.usda.gov/java/reference?symbol=CAPR)[2].

A popular source of research for students, Wikipedia, reports the re-categorization of *Calotropis procera* to be in Apocynaceae instead of Asclepiadaceae. The USDA website still classifies *C. procera* to be in Asclepiadaceae. Sinha and Mondal[3] studied the diversity of pollinia morphology in six members of Asclepiadaceae. They clearly recognized that the pollinial character is the most important taxonomical tool in identifying the specific genus in Asclepiadaceae (Dicotyledons) and in Orchidaceae (Monocotyledons). Detailed survey on pollinaria in Asclepiadaceae by Gaykar *et al*. confirm *C. procera* to be in Cynanchoideae of Asclepiadaceae[4]. Taxonomical re-categorization takes place all the time, but it is not clear why *C. procera* has been moved away from the sub-family Cynanchoideae of Asclepiadaceae. Presence of pollinia attached to the acellular translator apparatus made up of corpusculum, lateral blades, and retinacula is the main characteristic of Cynanchoideae[5]. Sreenath *et al*. reviewed 53 species with pollinia (some with spoon shaped carriers and others with the translator apparatus) to study evolutionary trend in the complexity of structures to transport pollen in Asclepiadaceae. They reported extensive diversity in the size, shape, orientation, extent of development, and nomenclature of carriers in species belonging to the subfamilies - Periplocaceae, Secamonoideae and Asclepiadoideae. They concluded that the simpler pollen-carriers (not translator apparatus) of Periplocaceae are primitive in organization and are similar to Apocynaceae and thus might be closer in phylogeny[6]. Based on taxonomic studies Huber[7] concluded that "Both Periplocaceae and Asclepiadaceae are derived from Apocynaceae. They are not closely related to each other than to Apocynaceae – Apocynoideae. He further concluded that the pollen carriers of Periplocaceae are closely related to Apocynoideae of Apocynaceae, while the pollinial apparatus of Asclepiadaceae has close relationship with the Periplocaceae and not with Apocynaceae." Thus, there is an evolutionary trend of diverse pollen carriers from *Apocynum* to Periplocaceae but there is no independent origin of Periplocaceae and Asclepiadaceae from Apocynaceae. DNA analysis is playing an important role in re-organizing relationships and classifications of plants. Reading about *C. procera* in Apocynaceae was another reason that I wanted to write this article to emphasize that DNA barcoding should be done with utmost precision by taking into account morphological, anatomical, and embryological features before the final re-categorization of any genus. Using DNA Subway, FASTA file of *Apocynum* from Apocynaceae will be compared with those of *C. procera* and *Asclepias syriaca* from Asclepiadaceae and *Phalaenopsis* of Orchidaceae. These files will be used to construct PHYLIP NJ tree to recognize the relationship among these families (http://dnasubway.i plant collaborative. org/[8]. We do need some order of classification in the living world based on morphology. Bioinformatics is helping to trace common enzymes or identical genes and it might bring down an artificial separation of living forms which are known presently to belong to specific Domains/Kingdoms/Families. In spite of common genes, plants struggle with local environmental stress leading to diverse morphological features that help them to achieve reproductive success. Co-evolution with insects and butterflies (to name some) has allowed angiosperms to dominate in the plant kingdom. Most flowers reveal the color that is visible to the pollinators. Others adapt to produce nectar or modify their shape to attract pollinators. Asclepiadaceae shows anatomical modifications in anther by making pollen aggregates to do mass transport of pollen because of a lack of pollinators in its dry arid habitat and the phytotoxicity of its vegetative parts. Plants are known to produce secondary metabolites as quantitative or qualitative inhibitors to prevent predation. Phytotoxicity of leaves in *Calotropis procera* has a great impact on herbivory, interspecific competition through allelopathy, and in causing destruction in croplands as a weed[9]. Pollinia or pollen aggregates could also be formed because of low pollen to ovule ratio. Another possibility is the self-incompatibility that exerts pressure on a plant to co-evolve with the pollinator. So, similar genes (or its variation) might result in the formation of a secondary metabolite to either form a simple exine or complex coating/translator apparatus. In addition to sharing excerpts from my thesis, I will share phylogenetic trees constructed using DNA Subway and NCBI site (National Center for Biotechnology Information - www.**ncbi**.nlm.nih.gov)[10] for Asclepiadaceae, Orchidaceae, and Apocynaceae.

Adaptive Features of *Calotropis procera*

C. procera, commonly distributed in tropics and sub-tropics, grows in waste lands with high salinity and drought or as a weed in crop fields (Fig. 1, 2). All parts of the plant such as root, stem, leaf, and flowers are used extensively to treat many ailments by indigenous tribes in India and many other Asian countries. These plant parts make an important ingredient of traditional medicines[11]. Yadav *et al*. have provided evidence for root extract of *C. procera* to have ameliorative effect in diabetic neuropathy which may be attributed by its multiple actions including as a potent hypoglycemic and antioxidant[12]. The rare visit of pollinator might have led to the formation of agglutinated mass of pollen to be covered with the secretion holding grains together. Further mutation while struggling to survive could have attributed some members of the family to secrete polymerized thick coating of complex secondary metabolite around the pollen grain mass and finally a hook with proper side blades to be carried by a bee during pollination.

C. procera shrub flowers during the spring or even winter in Southern India, has lavender flowers and contains thick milky latex. Condensed style and stigma form the gynostegium disc visible in the center of the flower (Fig. 2). Each of the five corners of this disc shows a black dot representing the corpusculum main body. One can insert a needle in the corpusculum and pull out a complete pollinium seen in Fig. 3. Pollinia in *C.*

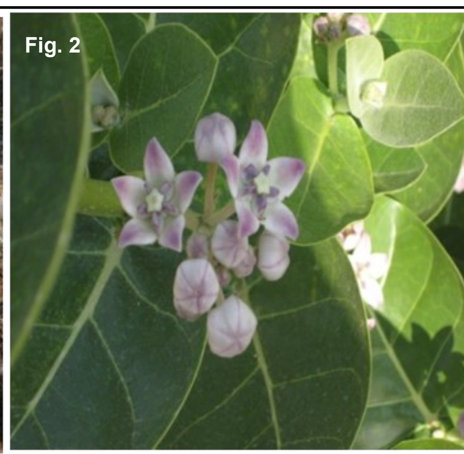


Fig. 3

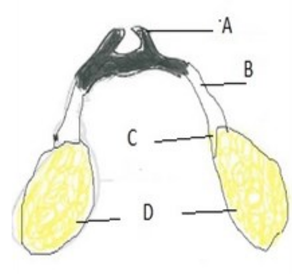


Fig.1. *C. procera* (January 2013, Bangalore, S. India). **Fig. 2**. Enlarged Flowers. **Fig. 3.** Digrammatic Pollinium with the Translator Apparatus (**A-** Corpusculum; **B-** Lateral Blade; **C**-Retinaculum; and **D-** Pollinia)

procera are carried by a complex structure where two pollen sacs are attached to the main body of the corpusculum. This corpusculum is extended on each side to form lateral blades ending in the swollen connective pads called retinacula. Each retinaculum connects lateral blade to the acellular beak of the pollinium (Fig. 3). The entire structure known as Translator Apparatus transports pollinia when hooked on to the pollinator. Specific co-evolutionary adaptations for pollinium removal and insertion during pollination in Asclepiadaceae have been attributed to the nectar production, location of storage, and collection by the pollinator[13].

Most plants produce pollen grains with specific ornamentation of exine which helps tremendously in recognizing sources of raw petroleum. In fact, palynology deals with the study of pollen grains, mainly exine with specific ornamentation and the protein that causes allergies to us. Exine is acetolysis resistant; it survives fossilization and is thermostable. The exine is composed of sporopollenin, a complex and highly resistant biopolymer containing fatty acids, phenylpropanoids, phenolics and carotenoids. In *C. procera,* exine formation is not as prominent as it is in the sub-family with the pollen grains. Histochemical localization of lipids, cutin, and phenol will provide insight into the nature of coating and translator apparatus to be sporopollenin or not. Following transportation of pollinium to the gynostegium disc, pollen tubes protrude out through an opening in the coating of pollinia.

Microscope Slide Preparation

The detailed study on *C. procera* included histochemical localization of metabolites such as polysaccharides, proteins, DNA, and RNA along with lipid, cutin and phenolic compounds during different stages of development to trace the origin and histochemistry of pollina and translator apparatus.

Instead of giving each and every detail about microscopic preparation of the slides, I will summarize the steps:

Floral buds collected at different stages of development were fixed in FAA, AA, and Carnoy's fixative.

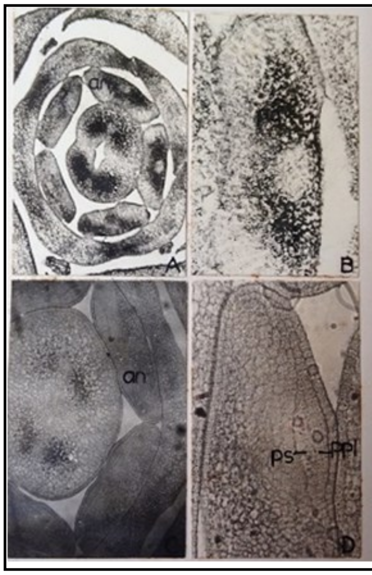
The specimen was processed through alcohol + xylene series, paraffin sections were prepared to stain with:

- Schiff's Reagent for Insoluble Polysaccharides (Periodic Acid Schiff's Reaction-PAS),
- Mercuric Bromphenol Blue for Total Proteins,
- Feulgen Reagent for DNA,
- Pyronin Y test for RNA,
- Sudan Black B test for Lipids,
- Sudan IV test for cutin,Ferric Chloride test for Phenolic Compounds.

Cross sections of floral buds were stained for specific metabolite, mounted on Canada balsam and were photographed using a home-grown photoscope that had a camera attached to the ocular tube of the microscope. It was the transitional time to move away from Camera Lucida and record pictures using a Camera fixed on the microscope.

Formation of Translator Apparatus and Pollinia

As seen in Plate 1, primary sporogenous tissue (Plate 1A-1D) lacks polysaccharide grains and directly develops into microspore mother cells



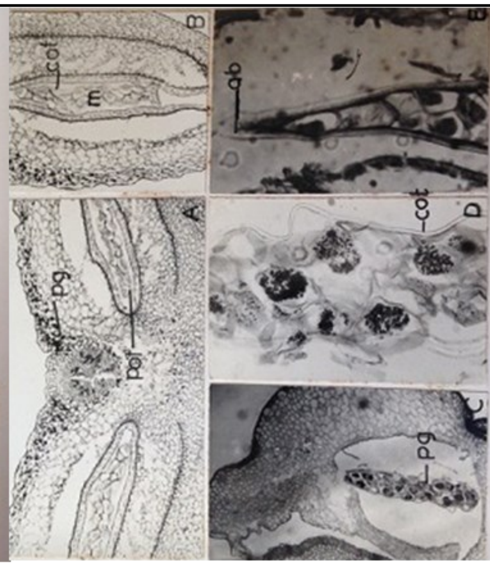


Plate 1A-1D (left). Early stages of anther development stain for Insoluble Polysaccharides.

Plate 2A-2E (right). Pollinia formation and mature pollen grains showing Insoluble Polysaccharide granules.

(Plate 2 A-2D). These mother cells divide to form microspores. Being metabolically very active, these cells do not show any insoluble polysaccharide grains. Insoluble polysaccharide grains (PAS grains) are visible in the tissue not directly related to microspore formation during early stages (Plate 1A-1D), whereas these grains appear in the microspores only at maturity (Plate 2A-2E). After formation of microspores, storage of insoluble polysaccharide grains clearly indicates slower growth phase (Plate 2A-2E).

Anthers are found in close proximity to the gynostegium disc (Plate 1A) which shows glandular cells similar to columnar epithelium (Plate 3A). These epidermal cells stain intensely for proteins during developmental stages. Radially elongated epidermal cells play an important role in the secretion of corpusculum (main body to be hooked by the pollinator) and lateral blades with the swollen parts (retinacula). During developmental stages, sporogenous tissue, microspore mother cells, and tapetal layer stain intensely for total proteins (Plate 3A-3D). Fully-formed corpusculum does not stain for proteins; lateral blades stain faintly whereas retinaculum stains densely for proteins. Proteinaceous retinaculum confirms its role as an adhesive pad that connects the main body of the corpusculum to the pollen sac. Protein concentration is much higher in the dividing microspore mother cells and differentiating microspores than in mature microspores. Nutritive tapetal cells around microspores stain intensely for proteins (Plate 4A-4D).

The translator apparatus (main body corpusclum, lateral blades and retinacula) are lipoidal in nature (Plate 5A-5D). The coating around the microspores also stains intensely for lipids at maturity (Plate 6A-6D). The translator apparatus shows some affinity for Sudan Scarlet IV revealing presence of cutin (Plate 7A-7D). Only the main body of the corpusculum stains positive for phenolic compounds, not the lateral blades or the coating in *C. procera* (Plate 8A-8B). Thus the composition of coating in *C. procera* differs from that of *Pergularia daemia* where the coating is phenolic in nature[14]. Schulze *et al*. found that highly purified exine fraction extracted from *Pinus* pollen clearly showed that different non-covalently bound phenols are located in and/or on the exine[15].

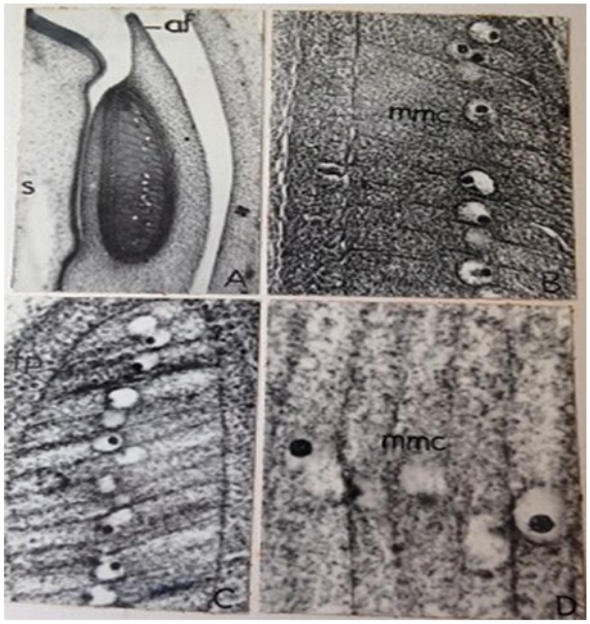
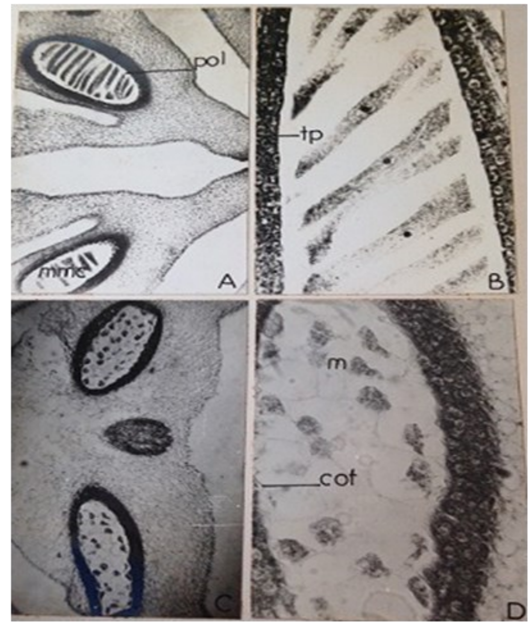


Plate 3A-3D (left). Early stages of anther development stained for Proteins.

Plate 4A-4D (right). Pollinia formation and Protein-rich tapetum; decline in protein content in mature pollen grains.



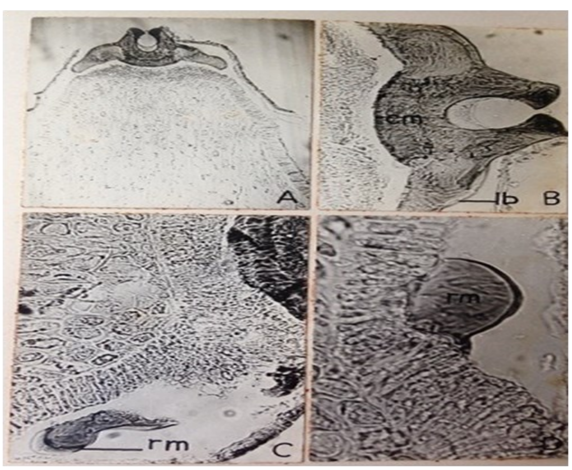
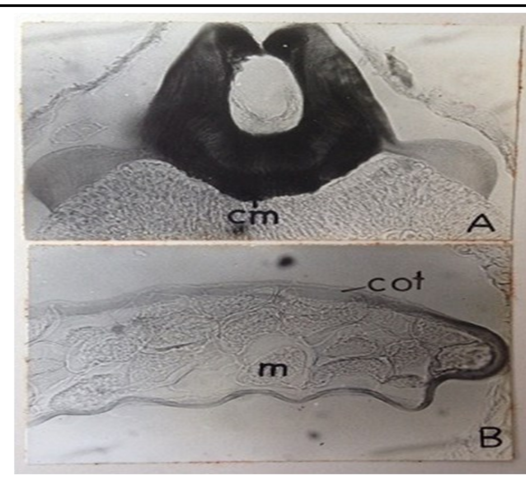


Plate 7A-7D (left). Corpusculum and Retinaculum stained with Sudan IV Scarlet for cutin.

Plate 8A-8B (right). Corpusculum and Pollinial Coating stained with $FeCl_3$ for Phenolic Compounds.

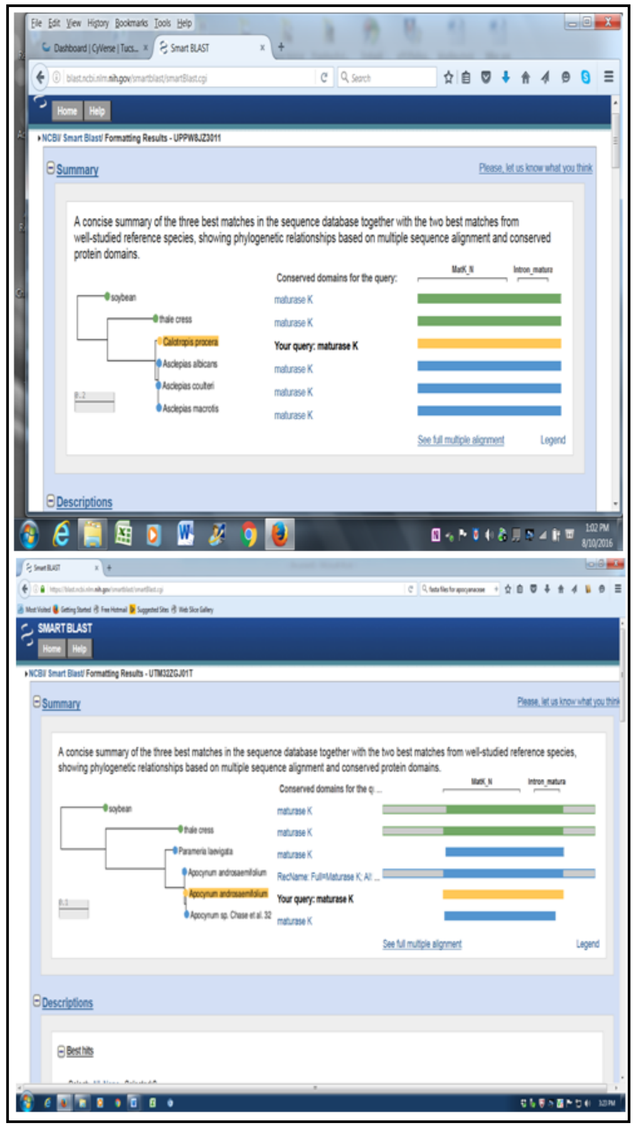


This study was undertaken to trace the ontogeny and histochemistry of the translator apparatus. During different staining processes, epidermal cells of the gynostegium disc were found to show abundance of proteins and nucleic acids. No insoluble polysaccharide grains were observed in these glandular epidermal cells. In *C. procera*, the primary stigmatic groove (of the gynostegium) clearly has a significant role in the secretion of translator apparatus which fits perfectly in the groove[16]. The secondary groove however, plays a secretory role for the formation of retinaculum. Kunze[17] also studied the ontogeny of the translator in three species from Asclepiadaceae and concluded that the corpusculum is a highly derived structure that seems to have taken its phylogenetic origin from the viscid liquid used as adhesive for the attachment of pollen in Apocynaceae. He further added that Asclepiadaceae members are defined by their possession of a solid translator which clings to the insect. Retinaculum, like corpusculum, is also stigmatic in origin for *C. procera*, but differs histochemically from the latter being composed mainly of lipo-protein complex and lacking phenolic compounds[18]. Tapetal cells secrete the coating around the pollen sacs. Diversity of pollinaria in Asclepiadaceae is extensive and there is no generalization that can be made about the extent of development of the carrier structures in this family[4]. Ulev has described *Asclepias speciosa,* a showy milkweed, found in US to have similar modifications of pollinia with the corpusculum[19].

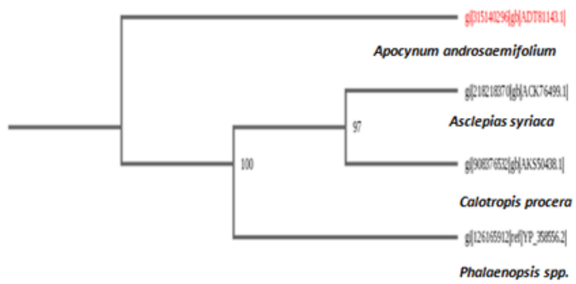
Two basic kinds of pollinia exist in Orchidaceae: One has soft, mealy packets bound together to a viscin core by viscin threads and is called sectile; the other kind ranges from soft, mealy pollinia, through more compact masses, to hard, wax-like pollinia; the latter usually have some mealy pollen with viscin strands that attach the pollinia to each other or to a viscidium. This portion of the pollinium is called the caudicle[20].

Bioinformatics and Family Relatedness

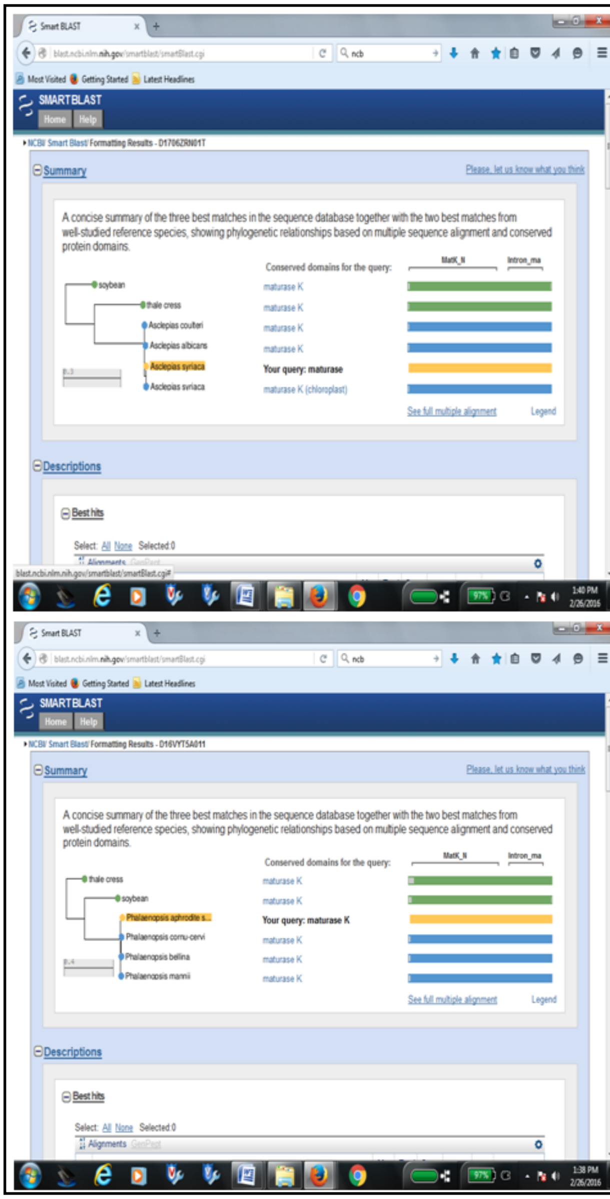
Not much has been worked out in relation to enzymes needed for pollinia or corpusculum formation, but with the common denominator of mutase K, I collected FASTA files. These FASTA files were processed through DNA subway and Smart BLAST. Surprisingly, *C. procera, Phalaenopsis* (an Orchid), and *Apocynum* (Apocynaceae) reveal an ancestral link to thale cress (mustard family) and soybean (bean family). The PHYLIP NJ phylogenetic tree constructed using those FASTA files for maturase K put Apocynaceae on a separate clade in relation to Orchidaceae and Asclepiadaceae. Orchidaceae belongs to monocots and Asclepiadaceae does not have much in common in morphology with Brassicaceae and Leguminosae. There are no inferences that can be drawn based on these phylogenetic trees at present, but mapping the sporopollenin



synthesis pathway might yield some meaningful connection among these families. Possibly, pre-disposed genes (for exine formation) found in angiosperm families become overactive to fight environmental stress and form complex structures. The purpose of this article is to promote organismal botany as the basis of molecular genetics and bioinformatics in solving taxonomical problems.



PHYLIP NJ Tree based on FASTA Sequences for *Phalaenopsis (Orchidaceae)*, *Calotropis procera* (Asclepiadaceae), *Asclepias syriaca* (Asclepiadaceae), and *Apocynum androsaemifolium* (Apocyanaceae).



Conclusions

In conclusion, to explain the diversity of pollinia and its carrier, one can only assume that plants, struggling to survive in adverse ecological conditions either because of physical factors or lack of pollinators or self-incompatibility, developed genetic blueprints to form these structures. Some species with less toxicity were probably able to co-evolve in mutualistic relationships without forming complex carrier (translator apparatus), while higher levels of phytotoxicity and environmental stress led others to form translator apparatus.

Acknowledgments

This paper is dedicated to my mentor, the late Dr. M. R. Vijayaraghavan whose passion for botany was contagious. I would also like to thank Prof. S. Beck for his support for Plants & Society and Molecules & Medicine courses at Nassau Community College.

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Changes in the Numbers of Butterfly Weed (*Asclepias tuberosa* L.) at Marshlands Conservancy in Rye, New York Over a 12 Year Period

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Abstract

Butterfly weed (*Asclepias tuberosa* L.) is a perennial plant mostly known as a food source for butterflies, including the monarchs. It has declined in numbers in New York State such that it is a protected by law as being "exploitably vulnerable." This study recorded the number of butterfly weed plants in an approximately two square kilometer area of a maintained meadow in Rye, New York from 2003-2014. After an initial increase in numbers from 2003-2005 to a high of over 1000 plants, there was a sharp decline of approximately 90% to just over 100 plants in 2008 and a further decrease to less than 50 plants in 2014. Hypotheses are presented as to the possible causes for this sharp decrease, including the increase in herbivory of this plant by white-tailed deer, decrease in the numbers of its pollinators, and climate change.

Introduction

Asclepias tuberosa L., also known as the butterfly weed or butterfly bush, is primarily known as a horticultural plant on which butterflies lay their eggs and later becomes a food source for butterfly larvae, including the monarch (*Danaus plexippus*)[1,2,3]. It is also known as "pleurisy root" and has been used over the years for medicinal purposes. For example, various Native American populations have used the root for lung and heart problems, to treat wounds, and as an emetic. European Americans have also used the root for lung and heart problems, as well as to treat pleurisy, a condition caused by an inflammation of the lungs and chest cavity[1]. Indeed, butterfly weed contains various chemical compounds that are poisonous to humans and livestock. Monarch butterflies have even evolved such that they have become tolerant of the toxins, and even express the toxins and use them as a defense against predators[1,3].

Butterfly weed is part of the milkweed (Asclepiadaceae or Apocynaceae) family. Unlike other milkweeds, however, it does not produce the milky sap that is indicative of this family[4]. Reproductively, *A. tuberosa* is self-incompatible and like another plant in its family, *A. exaltata,* may be so due to post-zygotic control at a single locus[5]. It is also able to reproduce asexually through rhizomes[1]. Butterfly weed is an attractive plant with clusters of flowers that range in color from yellow to orange to red. It has a broad range in the United States, mimicking that of the monarch butterfly, growing from Maine to Florida, and towards the west in the prairies and even to California[6]. The plants grow to a height of about two to three feet and is able to grow in a variety of habitats without need of much care, although it prefers full sun and well-drained soil[1,2,6,7]. Because of its beauty, ability to attract butterflies, and relative ease of maintenance, butterfly weed has become a desired horticultural product and is found in many butterfly gardens[1,2,7]. Based mostly on leaf morphology, three subspecies of *A. tuberosa* have been identified, with *A. tuberosa* ssp. *rolfsii* found mostly in the southeastern United States, ssp. *tuberosa* found along the eastern portion of the United States, and ssp. *interior*, the dominant form, found away from the coast and towards the Midwest and prairie states[4,8]. Unfortunately, in New York State this plant is disappearing rapidly from its wild habitat. Butterfly weed has been labeled "exploitably vulnerable" by the New York Heritage Program and is considered to be a "protected" plant under the NY State Environmental Conservation Law. "Exploitably vulnerable" refers to a non-rare protection category, where a particular plant species is "likely to become threatened in the near future…."[9,10].

In light of the rapid disappearance of these plants in the wild in New York State, it is crucial to gain a better understanding of the butterfly weeds' growth, reproductive habits, distribution, and genetic diversity, such that the plant can be preserved in the wild as well as in residential and commercial gardens. This is especially important considering the large decrease in numbers over the past few decades of the monarch butterfly, which relies on various *Asclepias* species, including *A. tuberosa*, for their survival[11]. In fact, there have been studies to suggest that one of the reasons why monarch butterflies have seen a decline in population may be due to the loss of milkweed host plants in the Americas, particularly in agricultural settings[11,12]. A study into the distribution of butterfly weed will not only shed light on its ability to survive and thrive in the wild, it may also be useful in the study of the ecology of organisms that rely on butterfly weed for their survival.

This study investigated the numbers of a population of butterfly weed plants in a maintained micro-environment. Specifically, this study has tracked the distribution of presumably native butterfly weed plants at the meadow at Marshlands Conservancy in Rye, New York over a period of 12 years, from 2003 to 2014. Marshlands Conservancy is part of the Westchester County Parks Department and is home to a number of habitats, including a forest, a meadow, and marshlands, which is known to house a number of different bird species. The meadow, which is maintained by annual mowing, has been home to a native plot of *A. tuberosa* for many years (A. Beall, personal communication, June 2003). It has been anecdotally noted that this plot has a diverse range of flower color for such a small area of land. This suggests that there is great genetic diversity within the butterfly weed plants in this meadow, making this area a useful environment in which to study these plants. Other *Asclepias* species also exist in this meadow, but at significantly smaller numbers.

In this study, we recorded a marked decline in this plant at this meadow for the time period studied, perhaps reflecting what is occurring in the rest of Westchester County and the state. Hypotheses are presented of what causes may be responsible for the plants' disappearance and any effects that may have.

Material and Methods

Plant distribution: Plants were surveyed in the meadow at Marshlands Conservancy in Rye, NY. Starting at the northernmost end of the meadow bordered by a path, 10x10 meter square quadrats of the meadow were blocked off with stakes. All *A. tuberosa* plants within each of the quadrats were identified and their relative positions noted. The information was compiled onto a larger map of the meadow (Figure 1). Approximately one-third of the meadow was mapped, varying slightly from year to year. However, there was a concerted effort to ensure that only the same area was counted from year to year and recorded as part of this study. In order to compare numbers from year to year, the southernmost end of a forested region within the meadow was used as the arbitrary southernmost boundary from which the numbers of butterfly weed were recorded in this study.

Note that when errors were made, particularly with the reading of the compass, the squares of the grid were adjusted on the larger map. Landmarks in the meadow were used to note errors. The quadrat sampling of *A. tuberosa* was accomplished from mid-June through early August in 2003, 2004, 2008, and 2014. If the plants were flowering, the colors of the flowers were also noted.

Results

For every year that butterfly weed was surveyed, the plant appeared to be prevalent throughout the meadow. For the most part, the plants seemed to be randomly arranged within the meadow, although there were areas where some plants seemed to cluster around one another, perhaps due to asexual reproduction through rhizomes (Figure 1). Most of the flowers were light orange, and some were a combination of colors (e.g. sepals one color, petals a different color), while others had flecks of darker red, but there was a range of flower color from yellow to dark orange. It was observed that the flowers changed color throughout the duration of the season, so it was difficult to determine the extent of the role of genetics on flower color determination. There was no distinction made among the three subspecies of *A. tuberosa.* Indeed, based on molecular evidence, it is unclear if subspecies actually exist[13].

In the years that were surveyed, there was a large fluctuation in the numbers of plants that were recorded. From 2003-2005, there was an increase in the number of *A. tuberosa* plants in the approximately two square kilometers of the Marshlands Conservancy meadow that was studied. In 2003, there were over 600 plants counted, while in 2004, over 900 plants were counted. The numbers seemed to stabilize in 2005, when over 1000 plants were recorded. Surprisingly, there was a sharp decrease of approximately 90% in the number of plants that were identified from 2005 to 2008, when only 107 plants were counted. There was a further decrease from 2008 to 2014, from 107 plants to approximately half that amount (Figure 1, Table 1).

It was noted that many of the plants from 2008 and 2014 were not in bloom, which made it very difficult to identify the species amongst a cacophony of flora. Without the distinctive orange flowers, there was no easy way to identify them. The percentage of plants in bloom were much less in those years than in 2003-2005 (Table 1). The lower percentage might account for the lower numbers recorded for those years. Interestingly, of

Figure 1. Locations of *A. tuberosa* plants at the Marshlands Conservancy meadow from 2003-2014 shows a marked decrease in the number of identified plants in recent years. Each dot represents the location of an identified *A. tuberosa* plant. Each square represents approximately 10x10 m^2. The meadow is bordered on the top and right by paths. The left border is a forested region. The shaded oval-like area in the lower left hand corner of the meadow represents a forested area where no butterfly weed can grow. Larger dots represent trees that were noted. Note that the locations of the trees were not always noted for every year. The numbers to the right of the meadow on the 2003 and 2004 diagrams represent the number of 10 meter rows. The greatest number of plants were recorded in 2005 and the least in 2014.

Table 1. Plant count of *A. tuberosa* in approximately 2 km^2 of the meadow at Marshlands Conservancy

	Summer 2003	Summer 2004	Summer 2005	Summer 2008	Summer 2014
No. of Plants Counted	628	920	1043	107	49
Percent of plants in bloom	78%*	95%	83%	11%	35%

*Based on plants in which data was available. Only one-half of the plants had data of having been in bloom or not.

a. b.

Figure 2. a. Photograph of *A. tuberosa* in full flower. b. Photograph of *A. tuberosa* in which the top of the plant appears to have been grazed, whereas the rest of the plant looks intact. Note that the plant is now being completely shaded by the plants that surround it.

those plants that were found in 2008 and 2014, most of them looked as if they had been grazed by herbivores (Figure 2). The lower portion of the plants (at least 30 cm from the ground) were intact, but it appeared that the tops of the plants were completely removed, preventing the plants from growing to full height, as well as from flowering and setting seed. *A. tuberosa*, a perennial, should not have declined so quickly. However, if the plants were constantly grazed, they would eventually die, and their numbers would decrease significantly.

Discussion

The fluctuation in the numbers of butterfly weed, from over 600 plants in 2003 to over 1000 plants in 2005 to under 100 plants in 2014, was surprising, considering that *A. tuberosa* is a perennial and should persist from year to year. From 2003- 2004, there was an increase of approximately 50% in the numbers of butterfly weed plants (Table 1). This increase may have been part of a steady increase that has been noted to occur over a number of years before this study was conducted (A. Beall, personal communication, June 2003). It may be that the plant started to take hold at the meadow at some previous point and steadily increased in number to the high recorded in 2005. Perhaps because a smaller percentage of plants were in flower as compared to 2004 and 2005, some plants may have not been identified and counted (Table 1). Because quantification was not performed previous to 2003, it is difficult to determine specific causes to the increase.

The most surprising result from this survey, however, was the sharp decline in the number of *A. tuberosa* plants in the meadow at Marshlands Conservancy from 2005 to 2008 and then to 2014, where counts went from a high of over 1000 plants in 2005 to less than 50 in 2014. A number possibilities exist that may explain this surprising decrease. For one, a high number of plants were grazed in 2008 and 2014, and it seems likely that deer were responsible for the herbivory observed on many of the plants. Deer populations have been known to reside in Marshlands Conservancy, and in fact, it was not unusual for researchers to spot deer when sampling for butterfly weed plants. Because the upper portion of the plants were grazed and cut off at the stem with lower portion of the plant intact and completely undamaged (including the leaves), it is unlikely that insects or other smaller herbivores, which would not be able to reach the height needed to cleave the tops, were responsible (Figure 2). In addition, other plants surrounding the *A. tuberosa* were not noted to have being eaten, therefore, the deer may have been selectively eating these particular plants.

It appears that the white-tailed deer population in New York State, and specifically Westchester County, has steadily increased in the last few decades[14,15]. The increase in the amount of deer herbivory may be significantly affecting the presence of this particular species of milkweed, and therefore, the ecology of this microenvironment. This is not unique, as deer herbivory has been implicated to have a significantly negative effect on the ability of New York forests to undergo regeneration, where increases in the deer population have decreased the number of seedlings and sapling required for forests to regenerate[16]. It is, therefore, plausible that an increase in deer at Marshlands Conservancy may have led to increased herbivory of butterfly weed, leading to a significant decline in plant numbers.

Herbivory of butterfly weed by deer is surprising, considering that the plant is known to synthesize chemicals that are considered to be toxic to other mammals, including humans and many livestock[1]. There has been a great deal of research on the ability of insect herbivores to tolerate and use *Asclepias* secondary metabolites, particularly the cardenolides, but there is a dearth of studies on the ability of mammals to tolerate them[17]. The apparent herbivory by deer may indicate that the plants are not as toxic to deer as compared to other mammals. Further studies might examine deer grazing behavior at Marshlands Conservancy to determine whether their selective herbivory includes *A. tuberosa* and whether the health of the deer are negatively affected by the consumption of these plants.

Another hypothesized explanation to the observed decrease in butterfly weed at the meadow may be due to the fact that the plant is primarily pollinated by bees and butterflies, both of which have also been steadily decreasing in numbers[11,18,19]. Without the pollinators, the plant may not be able to reproduce as quickly and, therefore, occupy the area in the same numbers. If this hypothesis is supported, it may be that the disappearance of bees and butterflies has a direct effect on the type of flora that is found in a particular area, changing the ecological balance of a microenvironment such as the meadow. Conversely, the disappearance of the milkweed plants may have a negative effect on the ability of the pollinators to survive, exacerbating the decline of all species involved.

A third hypothesis is that the plant in the meadow is being outcompeted by other introduced plants, particularly wild grape, which has been observed to have increased in certain parts of the meadow. In addition, there seemed to be an increase in the numbers of various types of grasses, which were observed to thrive in drier parts of the meadow. If that is the case, then changing climate conditions may influence the presence of the milkweed. In Westchester County, average daily temperatures did not

Table 2. Average daily temperature and precipitation for the month of July for 2003-2008, 2010, 2012, and 2014 according to WeatherUnderground.com.[20]									
	2003	**2004**	**2005**	**2006**	**2007**	**2008**	**2010**	**2012**	**2014**
Average temperature (Fahrenheit Degrees)	72°F	71°F	74°F	75°F	72°F	75°F	77°F	76°F	73°F
Average precipitation (inches)	0.55 in	2.41 in	2.64 in	1.49 in	1.08 in	0.58 in	1.27 in	0.42 in	2.27 in

significantly fluctuate over the time period in question (Table 2)[20]. However, the amount of precipitation did fluctuate, where the years with the highest precipitation, 2004 and 2005, correlated with the years in which the highest numbers were observed for *A. tuberosa.* In addition, there a relatively large drop in precipitation from 2006-2012, when the numbers of *A. tuberosa* also declined (Table 2)[20]. Climate change has been implicated in having an effect on *Asclepias* species distributions throughout the United States, which in turn, may affect the distribution of monarch butterflies[21]. It very well may be that climate change, in the form of differences in rainfall, is affecting the presence of this particular milkweed plant in this microenvironment.

It is likely that a complex combination of factors are causing the decrease in numbers of *A. tuberosa* in this meadow and that different factors may influence each other. For example, herbivory of the plant by deer may initially prevent the plants from flowering, which may then reduce the number of pollinators, which in turn would decrease plant numbers further and reduce the number of pollinators further. The presence of the deer or the effects of climate change may also alter the ecological conditions of the meadow, making it less favorable for the butterfly weed to prosper, which would, in turn, reduce pollinator numbers, reducing plant numbers further. Although this is a study on only one site, increases in deer population and decreases in bee and butterfly populations are not limited to this site, indicating that this plant may have similar fates in other areas of New York State, endangering this plant even further. It may be that native populations of *A. tuberosa* may need to be even further protected in this state.

One way to increase numbers of butterfly weed is to plant them in gardens, as it is a horticultural product. One study has shown that butterfly gardens may be effective in restoring habitat for the monarch butterfly, and various governmental agencies have promoted planting butterfly gardens to promote monarch butterfly growth[22,23]. Perhaps protections can include planting butterfly weed in deer-excluded gardens, allowing the plant to prosper, which in turn, would allow pollinators like the monarch butterfly to take advantage of a food source and increase in their numbers as well. However, native plots of butterfly weed, which may have its own unique genetic make-up may still be lost in the wild unless conservations efforts that target this plant are implemented.

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