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TEXAS SPECIES RESEARCH IAC FINAL REPORT

INTERAGENCY COOPERATION CONTRACT

(IAC) # 16-5998LV

Evaluation of the Status of the Monarch Butterfly, *Danaus plexippus*, with an emphasis on its current populations, host plant availability, and disease threat in East Texas

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FINAL REPORT

I. Stated Tasks and Deliverables for Project.

Our stated deliverables per the Interagency Cooperation Project are as follows:

1. Evaluate the correlation dynamics of the gregarine *O. elektroscirrha (OE)* and the monarch butterfly in east Texas.
2. Evaluation of red imported fire ants impact on monarch butterflies and whether red imported fire ants are a factor in eliminating part of the monarch butterfly population in east Texas. Additionally, if present, document if phorid flies are able to mediate the negative influences and fire ant predation.
3. Comprehensive list of all monarch butterfly natural enemies, including determined distributions of these species, their relative abundance in association with monarch butterflies, and an evaluation of the negative effects of each natural enemy.
4. Survey and evaluate results on the impact of introduced tropical milkweed, *Asclepias curassivica*, on monarch butterflies. This will include an evaluation as to whether this phenomenon is causing increased exposure to *OE* and natural enemies that significantly reduce monarch butterfly populations
5. Documentation of milkweed species of east Texas, along with their distribution and methods for identification.

II. Report on Deliverables

1. Dynamics of *OE* and the monarch butterfly

To address the dynamics of OE we conducted the study outlined in our original proposal. This study was headed by Co-PI Dr. Tamara Cook and her graduate student David Shaffer. The project was assisted by several Sam Houston State University students who were supported as part of this project. Below is the result of this study, which we hope to publish in the scientific literature in the future. Additionally, we surveyed monarch butterfies to document pathogen levels of migrating butterflies in both spring and fall migration. These results follow the report of the experimental infection study.

Correlates of Neogregarine Parasite (*Ophryocystis elektroscirrha*) Infection and Larval Monarch Butterflies (*Danaus plexippus*) Reduction in Growth

Introduction

Migration is a necessary life-history trait for many species where escape from some ecological pressure gives a significant selective advantage by allowing for increased reproductive success (Staerns 1976). It may be either obligate (migration between two places regardless of potentially acceptable niche space in or between those two places), or facultative (migration distances reduce or extend to ensure acceptable ecological conditions are encountered). Migration is distinguished from mobility and dispersal by its regular and predictable patterns and a tendency to operate at the population level (Dingle and Drake 2007).

Despite providing benefits, migration is difficult and energetically costly. Therefore, anything that makes migration more difficult, such as aberrant weather patterns, loss of stopover and terminal habitat, or infectious disease, is potentially devastating to migrating animals (Altizer *et al.* 2011; Bowlin *et al.* 2010; Chapman, Reynolds, and Wilson 2015). The monarch butterfly (*Danaus plexippus*) is a migratory animal facing the added challenge of infectious disease from the protozoan parasite *Ophryocystis elektroscirrha* (Bradley and Altizer 2005).

Danaus plexippus

The monarch life cycle consists of 8 stadia (egg, 5 larval instars, pupa, and adult). On average eggs hatch after 5 days and each larval instar lasts 2-4 days. Larvae reach the pupal stage in about 2 weeks and eclose after another 2 weeks. Adult monarchs have been known to live almost a month making their total lifespan about 2 months (Altizer and Oberhauser 1999; Zalucki 1987). The eastern North American population of monarchs performs a yearly mass migration from its overwintering grounds in the volcanic mountains of central Mexico to various feeding grounds as far north as Canada (Urquhart and Urquhart 1978). One migration cycle spans 5 generations of monarchs. An overwintering generation breeds and dies in Mexico. The offspring of the first generation as well as the next 2-3 generations travel northward breeding and depositing eggs on available milkweed host plants (*Asclepias* spp. L.). At the northern limit of their breeding range, exposure to cold temperatures causes the 5th generation to return to Mexico without stopping to breed. When the butterflies of the 5th generation reach central Mexican mountains they become the new overwintering population forming huge aggregations on native pine trees for protection against the elements (Urquhart and Urquhart 1978; Brower 1995). While this is the general pattern of monarch migration, some studies have reported a small number of 5th generation monarchs recolonizing the North along with the offspring of their peers rather than dying in Mexico in the spring (Davis and Howard 2005; Malcolm, Cockrell, and Brower 1993). Monarch migration is perhaps more facultative than obligatory as many populations of monarchs which live in areas with mild winters (e.g. Houston, Hawaii, and Florida) do not migrate (Personal observation, Leong, Yoshimura, and Kaya 1996; McLaughlin and Myers 1970). Continued monarch migration faces many threats including reduced winter habitat, decreased availability of host plants along their migratory corridor, and the spread of a neogregarine parasite *Ophryocystis elektroscirrha* (often referred to by monarch enthusiasts as OE) (Brower 1995).

Ophryocystis elektroscirrha

Ophryocystis elektroscirrha, a protozoan parasite in the phylum Apicomplexa, was first described in 1970 by McLaughlin and Myers from a population of queen butterflies (*Danaus gilippus*) in Florida. *Ophryocystis elektroscirrha* is an obligate intracellular hypodermal parasite of the monarch and queen butterflies. Larvae become infected with OE when they consume spores of the parasite deposited on their host plant (*Asclepias* spp. L.) by adult butterflies. Once inside the host gut, spores excyst releasing their primary multiplicative stage: micronuclear schizonts. Schizonts proceed to infect hypodermal cells where they reproduce via schizogony, eventually destroying the host cell. As host larvae pupate, schizonts develop into micronuclear merozoites which may be found in large numbers 5-10 days post pupation. About 8 days post pupation, macronuclear merozoites begin to appear, associate for sexual reproduction, and develop into gametocysts. Gametocysts undergo 3 nuclear divisions to produce a sporocyst with 8 nuclei. Each sporocyst develops into a single amber spore positioned on hypodermal cells fated to become exterior scales of the butterfly's abdomen after eclosion. From the exterior of the host abdomen, spores are eventually transmitted to the butterfly's host plant (*Asclepias* spp. L.), likely during the process of oviposition, where they lie dormant until being consumed by host larvae (McLaughlin and Myers 1970).

More than 20 years after the life cycle of OE was described many questions remained about OE, including: how long spores are viable, how many spores does it take to infect a larva, are most spores deposited on host plants during oviposition or by random perching, and is OE harmful to its host. A study done in 1997 began to answer these questions. The study demonstrated that early instar larvae were more susceptible to infection than later instar larvae, supporting the idea of vertical transmission during oviposition. The study also confirmed that spores passed through feces were uninfective, and year old spores were less infective than fresh spores, further supporting the vertical transmission hypothesis. Finally, this study demonstrated that adult spore load was directly proportional to initial spore dose and larval instar at infection (Leong *et al* 1997). This was a good start in answering questions about OE but it failed to address one of the biggest questions; is OE harmful to its host?

Two years later Altizer and Oberhauser (1999) determined that light infections with OE (spore load<1,000) have very little effect on the fitness of adult butterflies. They also determined that heavy infections (spore load>1,000) reduce adult fitness by decreasing rate of survival to eclosion, reducing wingspan, lowering adult body mass, and decreasing male lifespan and fecundity. However, while heavily infected females did have reduced lifespan and activity level, as well as increased adult weight gain, they did not show a significant decrease in fecundity which left the fitness cost of OE somewhat in doubt (Altizer and Oberhauser 1999). Another study, conducted in 2005, provided further evidence to support OE as a fitness-reducing factor for monarchs. The study focused on the flight abilities of heavily infected vs uninfected monarchs. Heavily infected monarchs had reduced flight speed and duration and lost more body mass covering the same distance when compared to uninfected butterflies. These factors were taken as indications of reduced migration ability in heavily infected monarchs, potentially supporting a "migratory escape" mechanism in monarchs (Bradley and Altizer 2005). In "migratory escape", individuals with reduced migration ability due to an infective agent are often removed from the population during migration thereby lowering the risk of infection to healthy individuals (Folstad *et al* 1991). Bradley and Altizer's 2005 study also found a higher rate of water loss in heavily infected monarchs, confirming earlier observations about water loss in heavily infected versus uninfected butterflies (Bradley and Altizer 2005, Leong *et al.* 1992).

Over the next decade further studies indicated that virulence (parasite induced mortality) in OE increases as initial spore dose increases, is increased when 1st instar larvae are infected rather than later instars, and can be reduced by the toxins contained in the host's chosen food plant. Ultimately however, virulence seems to be controlled by genetic factors in different strains of OE, which likely confer selective advantage through increased parasite transmission associated with increased virulence (de Roode *et al* 2007, 2008, 2009).

Knowledge about OE and how it interacts with its monarch host has increased greatly over the last two decades, but a blind spot remains. Despite a likely connection between damage done to larval stages and reduced adult fitness, almost no attention has been given to the impact of OE on the larval stages of the monarch butterfly (Leong *et al.* 1996; McLaughlin and Myers 1970).

The goal of the present study was to to determine what impacts OE has directly on the growth of larval monarchs that could lead to reduced fitness.

Objectives

I studied the effects of OE infection in larval instars of the monarch butterfly. My study focused on determining if increased infection with OE reduces fitness in larvae by reducing measures of growth. Growth was measured with three larval metrics: daily weight gain, daily length increase, and stage duration. Reduced growth, which is equivalent to delay of migration, was presumed to indicate reduced fitness. Data was also collected for larval death rate and adult spore load which have known relationships to increasing initial spore dose. Conformity to these known relationships helped to confirm that extraneous variables did not impact my results. An attempt was also made to gather data about the number of spots each subject developed as a pupa (another metric commonly referenced in the literature), but these spots proved difficult to objectively distinguish from normal pupal coloration and will therefore not be included in this study. Using these metrics, I tested the null hypothesis: daily larval weight gain, daily larval length increase, and time to pupation will be the same across all levels of initial spore dose (ISD), while larval death rate, number of pupal spots, and adult spore loads conform to expected relationships with increasing ISD.

Materials and Methods

Spore collection

Spores were collected from the abdomens of adult monarchs from the eastern migratory population of North America captured during their spring migration through Texas from sites including the Sam Houston State University campus, Waterwood limestone prairie, and Daphne prairie. Collections from migratory populations were supplemented with collections made from nonmigratory populations identified in Houston. Spore collection methods follow Leong *et al.* (1997). Adult butterflies were stored in glassine envelopes at 5˚C for no more than one month before spores were collected. Spores were collected by removing the abdomens of the butterflies and placing them in 2 ml plastic microcentrifuge tubes with 1.8 ml of 0.05% (v/v) wetting agent (Tween 20) in deionized water and washing for spores by agitating with a vortex mixer for 1 min, hand shaking for 10 min, letting stand for 2 min, and reagitating in the vortex mixer for a final 1 min. The abdomen was then removed and the wash suspension centrifuged for 10 min at 3000 rpm with a bench-top centrifuge. The supernatant was then discarded, 0.2 ml of the 0.05% wetting solution was added to the remaining suspension, and the suspension was agitated for 2 secs with a vortex mixer to resuspend the pellet. The number of neogregarine spores/ml recovered was determined with a hemocytometer and additional wetting agent was added to create solutions of the desired concentrations (500, 1000, 2000spores/10 µm). Control solutions containing no spores were produced by following the above procedures, substituting the abdomen of an uninfected monarch.

Experimental Protocol

Leaves of an appropriate host plant (*Asclepias curassavica*) were cut to 2 cm^2, wetted with 10 µmof the appropriate concentration of spores, allowed to dry and placed in plastic petri dishes. Eggs of the monarch butterfly were added to the petri dishes, allowed to hatch and consume the entirety of the cutting before being transferred to their treatment cage. Control group larvae consumed 2cm^2 cuttings wetted with 10 µmof the control solution.

Monarch larvae used in this experiment were hatched from eggs obtained from Shady Oaks Butterfly Farm, a Florida based butterfly farm, to ensure an adequate number of uninfected individuals for the experiment and to reduce larval genetic variation. Larvae were randomly assigned to treatments (0, 500, 1000, or 2000 spores) of *Ophryocystis elektroscirrha* wild caught from the spring migration of the eastern North American migratory population of monarch butterflies or residential populations from the Huston area. A larvae were assigned to each treatment. Each larva was raised on live plants of *Asclepias curassavica* surrounded by a mesh cage. Each cage was randomly assigned its treatment group to control for the effect of cage placement. Larvae were examined daily to ensure accurate stage information was kept and were measured daily to provide a record of weight and length changes. Larvae were weighed on a digital table top scale accurate to 0.001 grams and length was measured using a clear plastic 15 cm ruler. Information was also kept on mortality in each group during the larval and pupal stages. After eclosion adult spore load was calculated with a hemocytometer as described above.

An initial experiment done in January of 2018 experienced high levels of early stage larval mortality producing results that lacked appropriate sample size to produce confidence. This initial study was treated as a pilot study and a second experiment was performed in April of 2018 following the same methods with the exception of infection methods. Due to the author's travel schedule at the time infection was delayed until individuals had reached their third instar. All other methods remain the same.

Statistical analysis

Three 1-way ANOVA tests run in SAS (GLM procedure; SAS, 1985) were used to determine the presence of a treatment effect (ISD) on daily weight change, daily length change, and or time to pupation.

Results

Pilot Study

While lacking appropriate sample size (n_{isd0}=1, n_{isd100}=2, n_{isd500}=2, and $n_{isd1000}$=2) to produce confidence, the pilot study did not show a significant relationship between initial spore dose and: daily weight change (F= 1.33, P=0.342, df=3), daily length added (F= 2.11, P=0.1878, df=3), and stage duration (F= 0.76, P=0.5523, df=3). Within the highest dosage group all individuals surviving to adulthood were deformed, and the majority of pupa did not survive to eclosion. The second highest dosage group also yielded adults with deformities and malformed pupa which did not survive to eclosion. Neither the low infection group nor the control produced malformed pupa or deformed adults. This pattern of adult deformities and pupal malformities is consistent with the expectations of this study. The pattern of adult spore load also conformed to expectations with the individuals from the higher dosage groups having higher final spore loads.

Experiment Results

Although early stage losses remained a problem, the results of my second experiment seem to confirm the results of my pilot study with sample sizes that were much more appropriate (n_{isd0}=7, n_{isd100}=7, n_{isd500}=6, and $n_{isd1000}$=6). ANOVA testing failed to demonstrate a significant relationship between initial spore dose and: daily weight change (F= 2.22, P=0.0945, df=3), daily length added (F= 0.29, P=0.8290, df=3), and stage duration (F= 0.77, P=0.5127, df=3). Final spore loads were low when compared to analogous groups from the pilot study, but comparable between groups within this study. Adult spore load did not differ significantly between groups

(F=1.69, P=0.2371, df=3), however the heavy infection group continued to show the most adult deformities and pupal malformities followed by the moderate infection group, with no adult deformities in the light infection group or control group and only one pupal malformity in the light infection group. Several individuals also cleared their infection before reaching their adult stage. Clearing was seemingly most common in the light and heavy infection group with no clearing detected in the moderate infection group.

Discussion

While increasing initial spore dose has a clear effect on the pupal and adult stages of the host, there seems to be no significant effect on the larval stage which houses the primary multiplicative stage of *Ophryocystis elektroscirrha*. This is possibly due to a high turnover rate of the host's hypodermal tissue (the tissue which OE is most likely to damage in the larval stage) replacing tissue layers so quickly that even large amounts of damage have no effect. Given the very swift growth rate of these larvae (especially in the second experiment, larval stages lasted only a 3 or 4 days) compared to the pupal stages (in both studies these lasted at least 10 days) it does not seem unlikely that tissue turnover rate plays a part in the amount of damage retained from stage to stage.

It is also worth noting that environmental factors may play a major role in the transmission of this parasite. While it is possible that the low final spore loads and high incidence of infection clearing seen in my second experiment are related to infecting larvae at a later stage (Leong *et al* 1996), the chief difference between the first and second experiment was environmental temperature. The first experiment was begun in January amidst freezing temperatures which greatly slowed the growth of the larvae and killed many of them before their later stages where conditions improved. The second study began in late March (in Texas) when temperatures were beginning to rise. Early stages grew much more rapidly than the previous experiment going from egg to third stage larvae over the course of a weekend. Later stages were exposed to very high temperatures which seemed to stress the larvae and did seem to reduce growth somewhat. Almost all deaths after the egg stage for the second experiment occurred during the pupal stage with all surviving adults having low final spore loads. Attempts on the author's part to examine the pupae for spores post experiment were unsuccessful, but an interaction between spore load and heat stress does not seem unlikely.

Conclusion

While *Ophryocystis elektroscirrha* may not have any significant effect on the growth of larval monarchs its potential to harm populations of monarchs by reducing eclosion rates and increasing adult deformity remains strong. It also seems likely that the environmental factors surrounding monarch larvae infected with OE have an impact on the effect the parasite will have on later stages of its host. These impacts should be studied to gain a better understating of how this threat is likely to progress in light of changing climates and the altered migratory behavior of monarch butterflies.

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Documenting Pathogen Levels of *Ophryocystis elektroscirrha* in East Texas

Methods

A total of nineteen migratory butterfly collections were made to document the presence of *Ophryocystis elektroscirrha* (OE) in East Texas. Methods for non-invasive sampling were followed, which consisted of catching butterflies and using Scotch tape to sample for any *OE* present. The tape was mounted on a microscope slide and returned to the laboratory for evaluation under light microscopy (Fig. 1 & 2). Each of these sites were collected to get a minimum of 5 butterflies and sampling was halted if 20 butterflies were collected. The following were the collection locations included in the study in the order they were collected. The first ten sites were collected during the fall migration in 2016 and the last nine were collected in the spring migration of 2017:

FM 2550 and HWY 30, near Huntsville collection 1 (pupae)
FM 2550 and HWY 30, near Huntsville collection 2 (adults)
Nacogdoches, Texas collection 1 (pupae)
Nacogdoches, Texas collection 2 (adults)
Oak Drive, Houston, Texas
Thorton Rd., Houston, Texas
HWY I-45, near Houston, Texas
Daphne Prairie, near Mount Vernon, TX collection 1 (adults)
Daphne Prairie, near Mount Vernon, TX collection 2 (caterpillars)
SHSU campus, Huntsville, Texas
Waterwood Prairie near Lake Livingston collection 1
Waterwood Prairie near Lake Livingston collection 2
Waterwood Parkway near Point Blank
Mathews Lake, Huntsville, Texas
Oakhurst, Texas
Waterwood Prairie near Lake Livingston
Hunsville, Texas, May 20, 2017

Porter, Texas, May 21, 2017
Porter Springs, Texas, May 30, 2017

Figure 1: Adult Monarch reared from *Asclepias amplexicaulis* in Wood County, Texas in May 2017 has been taped to test for OE parasite and released. This wild caught specimen from a rural area on a milkweed that is occurs in style 2 was uninfected. The trend appearing is indicating that urban monarchs are more infected and rural ones are cleaner.

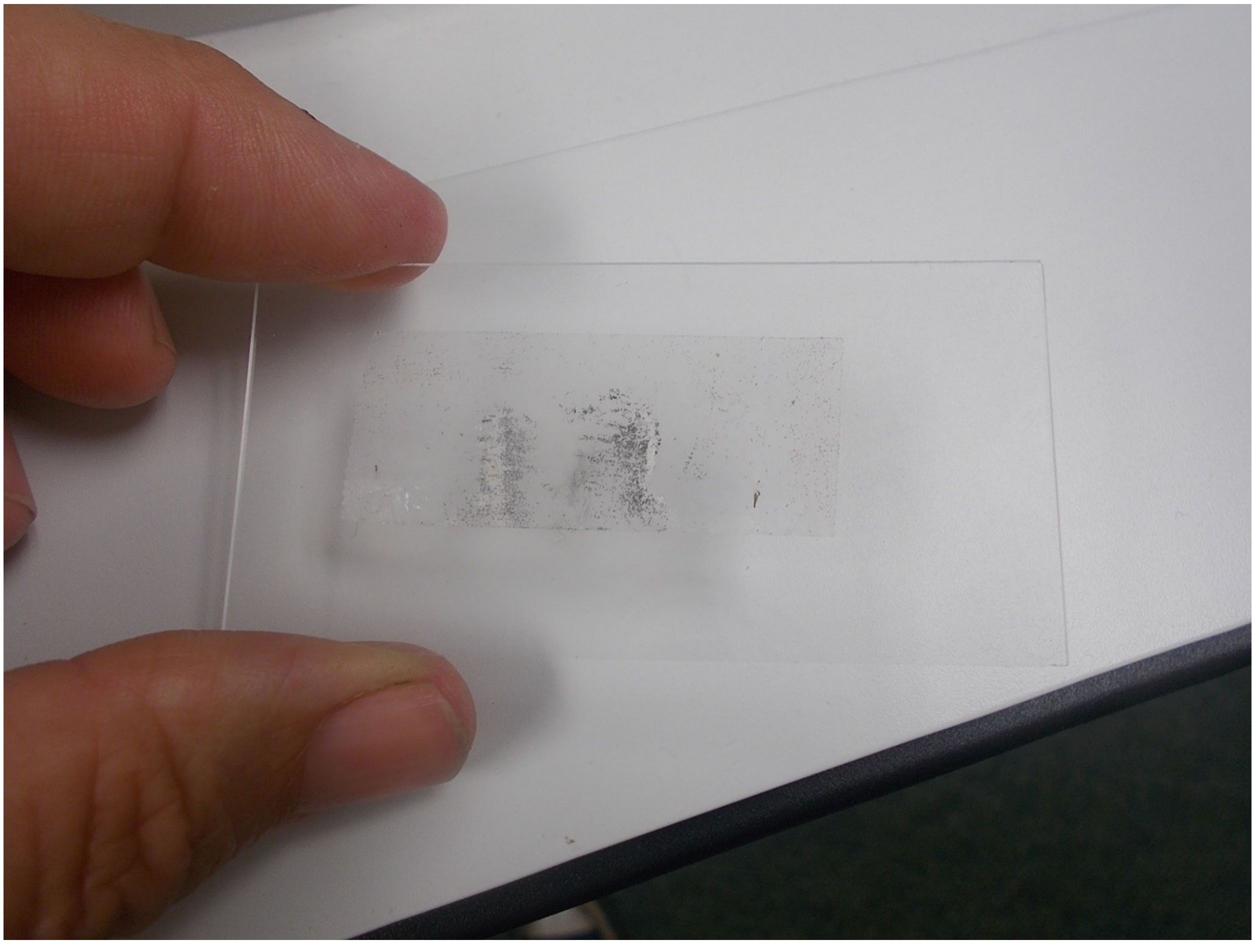

Figure 2: Taped Monarch abdominal scales are slide mounted and examined under the microscope for presence of the OE parasite.

Results and Discussion

The results of this survey are shown in Table 1. A general perception of these collections is that most migratory butterflies in east Texas were found to be free of *OE*. This was especially true of specimens where only native milkweed was present. Of the 17 collection sites where only native milkweed was present, two had butterflies with infections. Because tropical milkweed was not present we are confident that these were migratory specimens and not specimens staying in that locality year round due to the fact that native milkweeds are not present throughout the year to support monarch butterflies. At sites where the pathogen were found, most were not infected. This is exemplified by the collection at Waterwood Prairie on April 15 where only 14 percent of the butterflies collected were infected (2 out of 14 total). It is worth noting that the infected butterflies were always heavily infected in our collections. The specimens collected where tropical milkweed was abundant are probably not migratory as we will discuss later in this report. Thus, we found that there was only minimal infection of *OE* being carried in the migratory pathway of east Texas. However, the heavy infection found in some specimens shows that there is ample pathogen to fuel an infection under the right circumstances.

In areas where Tropical milkweed was common, we actually saw almost no native milkweed. These were primarily urban areas where most of the landscape is covered with managed plants. If milkweed is planted in southeast Texas it is almost always the tropical milkweed species. While it is not an absolute certainty that native milkweeds cannot be found in this area, we concluded that the butterflies collected in this area likely use the tropical milkweed as a host plant and likely do not migrate. In these specimens we found that most carried heavy infection but there were a small number that could be found that were not infected. It is possible that these represented migratory specimens that were simply making their migration through a region that was dominated by non-migratory butterflies. However, this is speculation and we have no way to document the origin of any of the specimens we collected for this part of the study.

Site	**Monarch Stage**	**Infection**	**Milkweed**
FM 2550 and HWY 30, near Huntsville	Pupa	No	Native
FM 2550 and HWY 30, near Huntsville	Adult	No	Native
Nacogdoches, Texas	Pupa	No	Native
Nacogdoches, Texas	Adult	No	Native
Oak Dr., Houston, Texas	Adult	Yes/No	Tropical
Thorton Rd., Houston, Texas	Adult	Yes	Tropical
HWY I-45, near Houston, Texas	Adult	No	Native
Daphne Prairie, near Mount Vernon, TX	Adult	No	Native
Daphne Prairie, near Mount Vernon, TX	Caterpillar	No	Native
SHSU campus, Huntsville, Texas	Adult	Yes/No	Native
Waterwood Prairie near Lake Livingston	Adult	No	Native
Waterwood Prairie near Lake Livingston	Adult	Yes/No	Native
Waterwood Parkway near Point Blank	Adult	No	Native
Mathews Lake, Huntsville, Texas	Adult	No	Native
Oakhurst, Texas	Adult	No	Native
Waterwood Prairie near Lake Livingston	Adult	No	Native
Hunsville, Texas, May 20, 2017	Adult	No	Native
Porter, Texas, May 21, 2017	Adult	No	Native
Porter Springs, Texas, May 30, 2017	Adult	No	Native

Table 1. Collections of *Danaus plexippus* to document the presence of infection by *OE.*

2. Impacts of the red imported fire ant on monarch butterflies

Our study on the effects of the red imported fire ant on the monarch butterfly is entirely observational as was stated in our proposal. We did not experimentally test if there were any effects, which is being undertaken by another researcher, who is not part of our program at Sam Houston State University.

Throughout or study, when we were doing field surveys, we watched for interactions between the red imported fire ant and monarch butterflies and their larvae or pupae. The short statement of our findings is that in the many hours we spent in the field observing and collecting *Danaus plexippus* in all its life stages, we did not observe any predation or results of predation. The entire region where we conducted our study has relatively large populations of fire ants, in some areas reaching over 100 mounds per acre. In at least one area (the Center for Biological Field Studies) near Huntsville, Texas we observed phorid flies at fire ant mounds. Still, in this area there are 20 to 40 mounds per acre.

Fire ants would rarely be expected to attach adult monarch butterflies and their only opportunity would be when they land. The larvae and pupae however represent stages that are not able to escape predation by fire ants. Our project collected and observed large numbers of pupae and larvae and we never witnessed any predation or signs of predation by fire ants. Having stated our observations, we realize that we were only in the field for a short period of time in the life of these monarchs and we were always collecting during the day when fire ants are not typically as active when temperatures are hot.

3. Natural enemies of the Monarch butterflies

As with all species, there are a large number of other organisms that prey upon or infect monarch butterflies. Only a few of these have the potential to do more than participate in the natural modulation of the species. The pathogen *EO* has been suspected of having the potential to dramatically impact monarch butterfly populations, although as shown above, this was not absolutely apparent in our study, nor was that of the red imported fire ant, which has been suspected to play an important role in decreasing monarch butterfly numbers (Calvert 2004). We did find that as monarch butterflies become non-migratory in some areas there can be an increase in pathogens, such as *OE*, and certain predators, such as *Polistes* wasps, but this does not appear to eliminate enough of the population to have a long term effect. Our observation of predation by *Polistes carolina* is the first report of predation of monarch butterflies by this species.

We observed several natural enemies and accumulated data from other sources to make a list of natural enemies of the monarch butterfly (Table 1). Monarch butterflies are not regularly preyed upon by birds, as are many lepidopterans, because they tend to be generally unpalatable. However some bird predators appear able to differentiate and consume some of the monarch butterflies that may be more palatable. Birds known to fit in this category include cardinals, some jays, some sparrows, robins, grackles, brown thrashers, orioles and the black headed grosbeak. Other vertebrates have been suspected (Smithers 1973) and mice have been documented to feed on monarchs, but this was usually only dead or moribund speciems (Brower et al. 1985). There are quite a few non-vertebrates that are enemies because of predation or parasitization. These are summarized in the Table 1. We especially note that some of these enemies are introduced species. It is likely that the native species listed have been natural enemies with a long association and are not likely to be the cause of the recent decline in the monarch butterfly. Still, when factors, such as habitat or host plant availability, change, then the magnitude of the effect caused by these natural enemies can also change. Introduced species should be viewed differently as they are a new threat that could be a contributing factor or a cause of monarch butterfly decline. Not all of the known natural enemies are found in east

Texas. In Table 1 we note which are in east Texas and which we observed during our field observations. Part of this list can be attributed to Oberhauser et al. (2015).

Natural enemy	Stage attacked	Introduced species
Red imported fire ant**	egg, larva, pupa	Yes
Formica ants	egg	No
Polistes dominulus	larva, pupa	No
*Polistes carolina****	larva, adult	No
Vespula alascensis	adult	No
Pteromalid parasitic wasps*	pupa	No
Lacewing larvae**	egg	No
Asian ladybeetle**	egg, larva	Yes
*Lespesia archippivora****	larva	No
Tachinid flies**	larva	No
Chinese mantis**	larva	Yes
Pentaomid (stink bug nymphs) **	larva	No
Crab spiders**	larva	No
Jumping spiders**	larva	No
Dragonflies**	adult	No
Ophryocystis elektroscirrha (*OE*)***	larva, pupa, adult	No

Table 1. Known non-vertebrate natural enemies of the monarch butterfly and the stages that are preyed upon. Species followed by * would be expected to be in east Texas. Those designated by ** have been observed by our study in east Texas. Those which we observed to parasitize or prey upon *Danaus plexippus* in east Texas are designated by ***.

Two of the natural enemies are of note as they were commonly observed predators of the monarch butterfly in east Texas during the time of our study. The tachinid fly, *Lespesia archippivora*, is a common natural enemy throughout the North American range of the monarch butterfly. The vespid wasp *Polistes carolina* is found throughout much of Texas as well as other parts of the United States Gulf Coast. Previously it has not been reported as a predator of monarch butterflies but it was commonly found doing so in our study.

We collected numerous larvae during our study and several of these were parasitized by *L. archippivora* (Fig. 3). Oberhauser et al. (2015) determined that if several of these fly parasites were found on a monarch larva, then the likely result was mortality of the larva. However, in cases where there one or a small number of fly larvae were parasitizing a monarch larva, it had a good chance of survival to produce a pupa and adult. In our observations, we most commonly found one or two tachinid larvae developing on its monarch larval host. We did not quantify this relationship or study the results of the parasitism we observed. It is suspected that the results of Oberhauser et al. (2015) in Minnesota would be similar to the relationship of these species in Texas.

Figure 3. The tachinid fly, *Lespesia archippivora.*

Photo from inaturalist.org

Figure 4. *Polistes carolina*, a predator of the monarch butterfly.

Throughout our study *P. carolina* was observed attacking adult monarch butterflies, at least in the sites that were presumed to be non-migratory populations of the monarch butterfly. This wasp species (Fig. 4) is an aggressive generalist predator. Around areas where tropical milkweed is grown, monarch butterflies are non-migratory. Therefore, the monarch population is constant in these areas and represents the largest population of a large insect that can become prey for *P. carolina*. This large population then becomes a primary food source for the opportunistic wasp. We did not see any evidence that the monarch butterfly is not palatable to the wasp. This association did not cause the population of non-migratory butterflies to decline during our study, in fact the monarch butterfly population appeared to grow during our study, probably due to the increase in amount of tropical milkweed being planted in this area. *Polistes carolina* is found throughout east Texas but we did not observe predation outside that area of tropical milkweed where populations of the butterflies were more dispersed.

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Calvert W. H. 2004. The effects of fire ants on monarch breeding in Texas. *In* K. S. Oberhauser and J. J. Solensky, eds., The Monarch Butterfly: Biology and Conservation, pp. 45-53. Cornell University Press, Ithaca.

Oberhauser, K. S., M. Anderson, S. Anderson, W. Caldwell, A. de Anda, M. Hunter, M. C. Kaiser, and M. J. Solensky. 2015. Lacewings, wasps, and flies – oh my. *In* K. S. Oberhauser, K. R. Nail, and S. Altizer, eds., Monarchs in a Changing World, pp. 71-82. Cornell University Press, Ithaca.

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4. Dynamics of overwintering populations of monarch butterflies due to the establishment of the tropical milkweed.

We identified four sites for our overwintering studies. Our original goal was to find three to five study sites. These sites have an abundant planting of the tropical milkweed, *Asclepias curvassavica*, that has been reported to encourage monarchs to stay in one region instead of migrating. At all of these sites we have observed monarch butterflies throughout the study time, including observing a butterfly that had been previously tagged (Fig. 5).

Tagged Monarchs in Herman Park are evidence of non-migratory populations persisting in urban environments. Monarch Watch received a request for tags from the Houston Museum of Natural Science on August 3, 2016. Tags were mailed on August 17 and the butterfly #WLU was tagges soon after. Two observations from these tags have been reported from Herman Park. Butterfly # WLU 228 was photographed on Nov. 21.

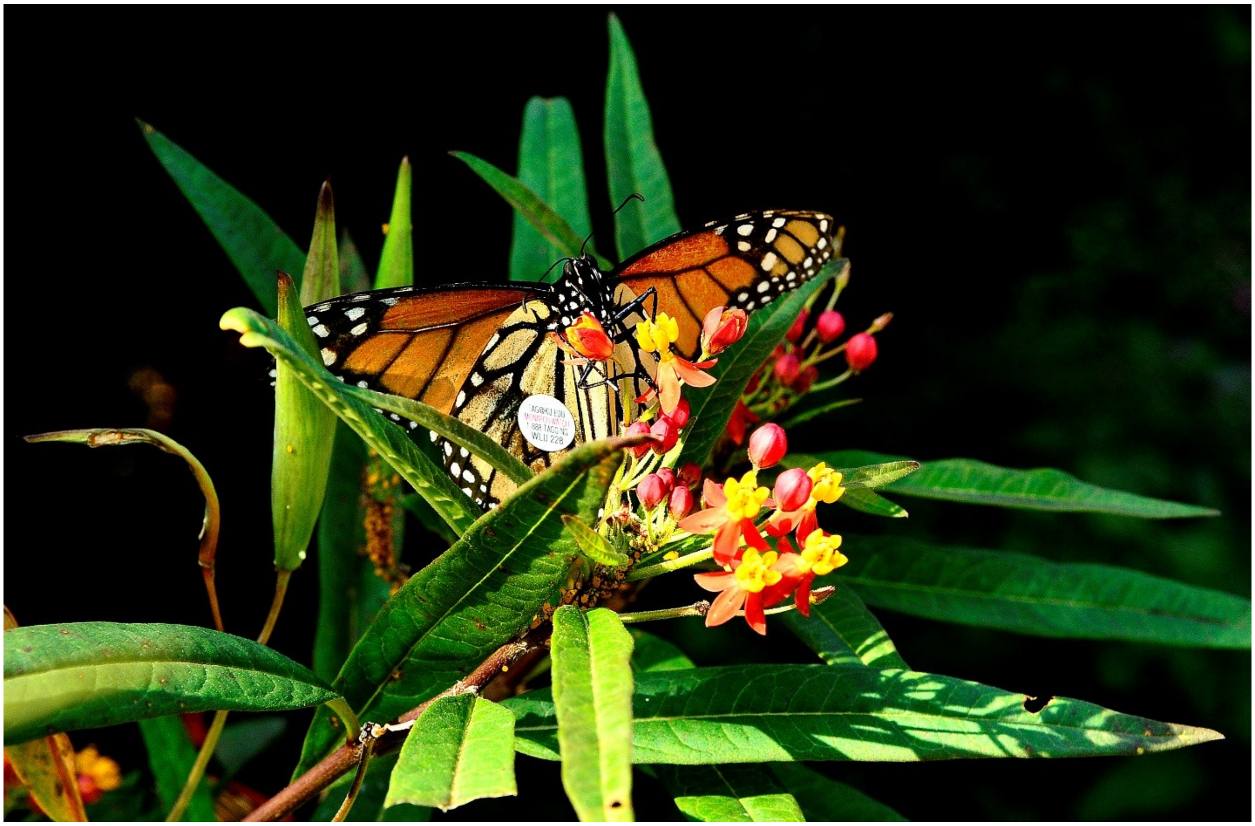


Figure 5: Tagged Monarchs at Herman Park persisting until well after migration has occurred in Fall 2016. These were observed up until November 28, 2016. Photo by Randy LeGrand.

The four sites used for our overwintering study are documented in Figures 6-11. The locations of the sites include Shangrila Gardens in Orange, Texas; Herman Park Gardens in Houston, Texas; the private property of the W. Fulton Broemer office, Houston, Texas; and gardens at the Private home of Lauren Simpston, Houston, Texas. The sites and contacts are as follows:

I. Shangrila Gardens, Orange, Texas Contact: Jennifer Buckner

Figure 6. Greenhouse and gardens at Shangrila Gardens.

II. Herman Park Gardens, Houston Texas, Contact: Jane Curtis

Figure 7. Monarch at Herman Park feeding on nectar from tropical milkweed. Photo by Randy LeGrand.

III. W. Fulton Broemer Office 3201 White Oak Houston, Texas

Figure 8. W. F. Broemer Office August 23, 2016

Figure 9. Monarchs at W. F. Broemer Office August 23, 2016

Figure 10. Gardens at W. F. Broemer Office August 23, 2016

IV. Lauren Simpson gardens 1228 Thornton St, Houston, Texas

Figure 11. Lauren Simpson Gardens, Houston, Texas, August 23, 2016.

We monitored the four sites established in our first year of the study for our overwintering studies. These sites continue to have an abundant planting of the tropical milkweed, *Asclepias curvassavica*. The tropical milkweed has even become more abundant in the Houston area and several public parks now contain this plant as do many residential areas. Although not due to any input from our project, Sam Houston State University has also incorporated this plant in its landscape. The plant is being sold by many commercial sources in the area and has become a popular landscape plant. At all of these sites where we have collected, we have observed monarch butterflies. This increase in tropical milkweed in the Houston region will likely continue to expand the number of non-migratory monarch butterflies. The effects of this on the species is uncertain.

As previously reported, all indications are that these monarch butterfly populations are non-migratory but appear to be an increasing population. There are also abundant red imported fire ants in this region and as was reported above, we have no observation or indication that fire ants are impacting these populations. The only difference we see between these and the natural migrating populations is that OE occurs in heavy concentrations in nearly all of these butterflies. We have sampled these populations continually for OE and it is rare that we find one without a heavy infection, yet no direct mortality has been noted. There was no indication that this heavy infection is dramatically affecting the natural population.

Over the winter of 2016/17, our partners in Houston, Texas made observations of adult Monarchs throughout the season, but in the winter 2017/18 no observations were made for a few weeks after the middle of January due to unusually cold temperatues. This recent winter differed by having a more severe cold event where temperatures in Houston dipped to the teens in mid-January (Fig. 12).

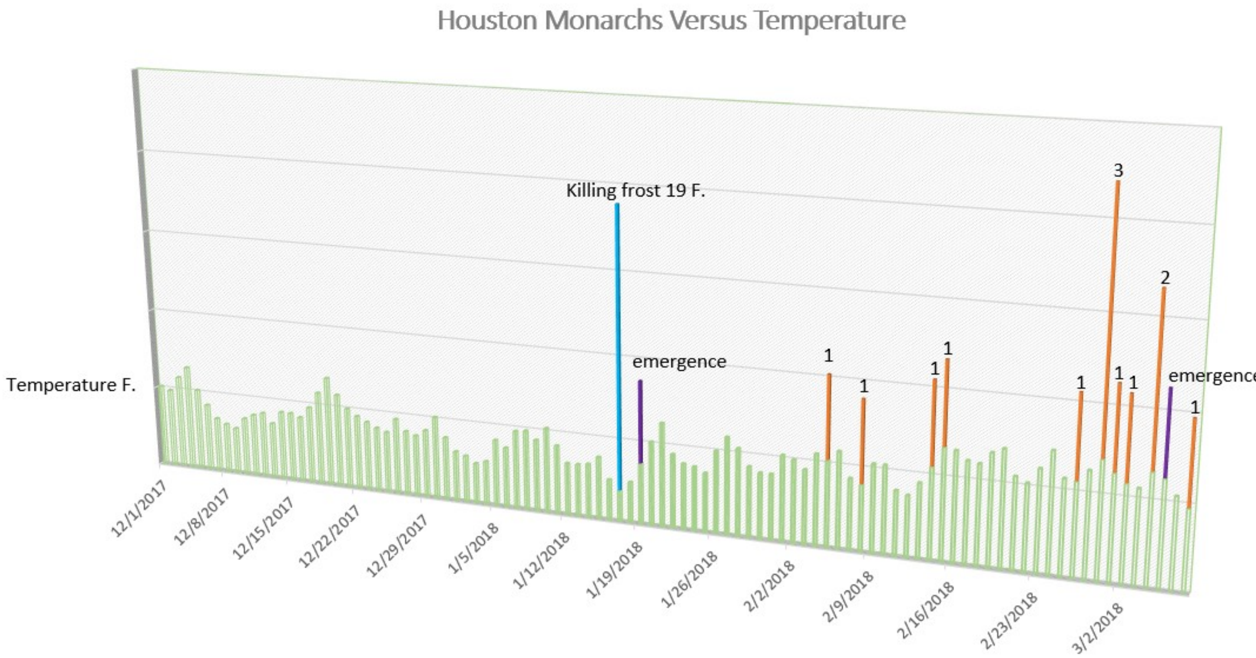


Figure 12. Temperature in Houston (green) dipped into a killing frost in Mid-January. After this hard freeze, Monarch butterfly emergences were observed at times shown in purple and adult monarch butterflies were observed at times shown in orange.

Monarch observations were recorded from Monarch Watch records and others participating with SHSU. While we did not see evidence of butterflies at our study sites, there were some unusual emergences from pupae in the winter noted at other sites, but even at these sites there was also a notable hiatus in monarch observations for 2-3 weeks following the freeze. We can assume that many of the observations in March are migrants from Mexico, but the endemic populations in Houston have persisted over the winter as pupae as evidenced by observed pupal emergences, although probably in greatly reduced numbers. We could expect parasite observations to show lower density in Spring 2018 if a die-off of overwintering adults is a factor although we saw no real differences in *OE* prevalence when the population returned to pre-freeze levels.

This freeze gave us a unique opportunity to better understand overwintering populations, especially since the planting of tropical milkweed is becoming more common in semitropical regions of the United States, like in southeast Texas. It is already expected that non-migrating populations build higher titers of *OE*, which might or might not have significant effects on monarch populations (our *OE* experimental tests suggest that there could be some effects caused by these increases but we are not seeing this reflected in natural population levels). This dramatic reduction of overwintering populations gave us the opportunity to see what this does to OE levels as well as the size of populations overwintering on tropical milkweed. The latter could be influenced by migratory butterflies encountering the tropical milkweed and becoming non-migratory. As for the status of the presumed pathogen (*OE*) there are questions that might be answered. Will an occasional hard freeze lower the build-up of *OE* concentrations, or is *OE* environmentally maintained in this environment once high levels are established by the overwintering populations? These answers have important consequences to managing monarch butterflies since substantial parts of the population may cease to migrate. Our limited study suggests that *OE* levels do not change after events such as these. Either the spores are resistant or the reduced survivors of these events maintain the pathogen loads.

There is little doubt that south Texas has a non-migratory population. What this means to the ultimate fate of monarch butterflies is unknown but presents several questions. Will some migratory butterflies continue to incorporate with the existing non-migratory populations, possibly decreasing the migratory portion of the species? Is there gene exchange between the migratory and non-migratory populations? Is there a long term detrimental effect of the formation of a non-migratory portion along the normal migration route of the monarch butterfly in the form of creating new predators or increasing *OE* prevanence? Is planting more tropical milkweed and establishing large non-migratory populations a method to conserve the monarch butterfly? These questions were beyond the scope of our current project but could be important future projects if our goal is to conserve the monarch butterfly.

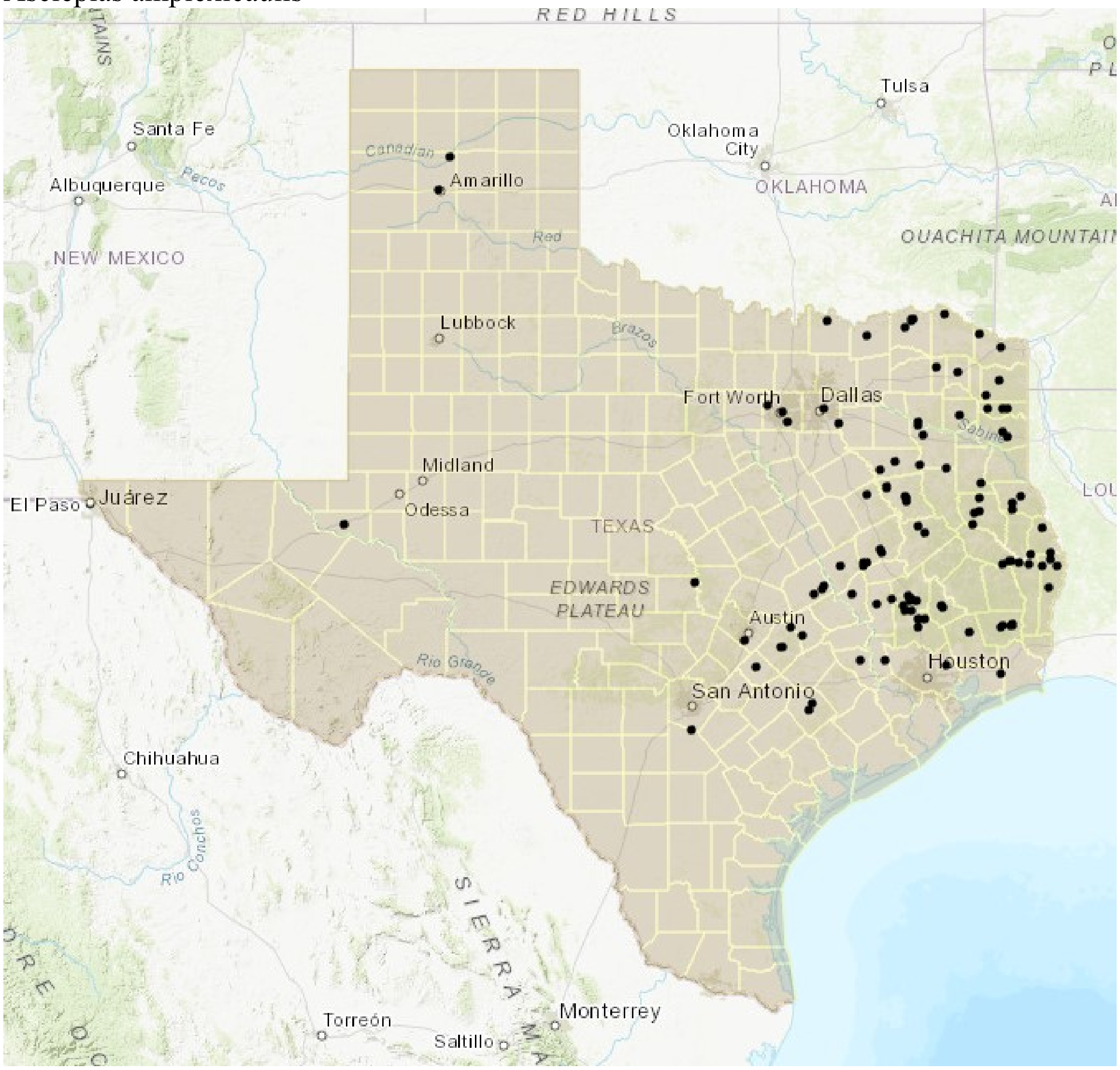
5. Milkweed species in Texas to support monarch butterfly populations.

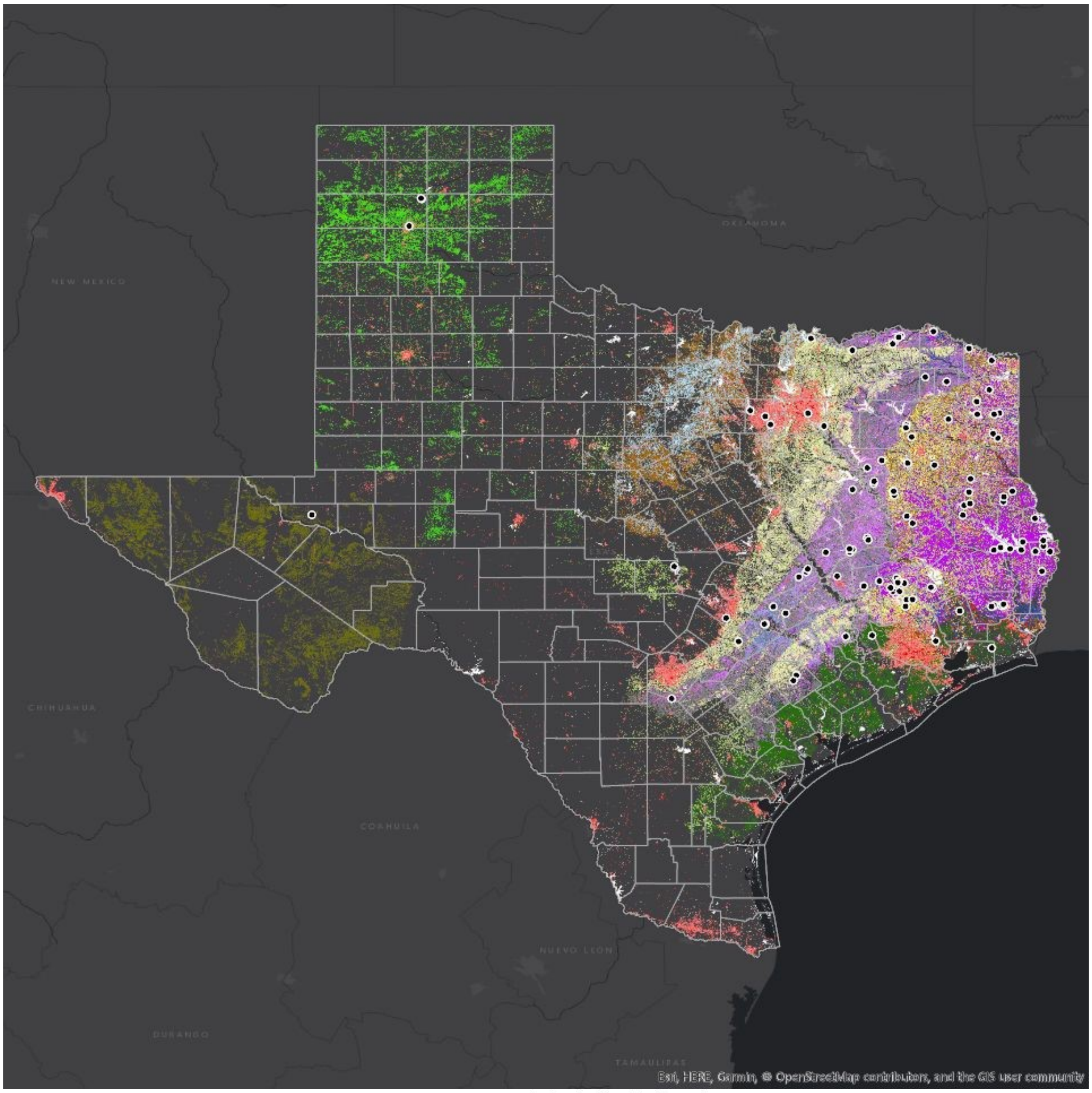
Asclepias of Texas

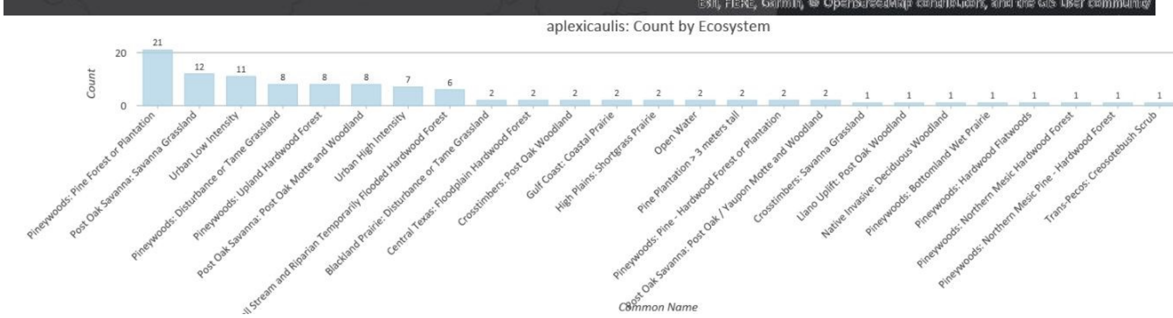
Each map is followed by an Ecosystems map and a histogram of the ecosystems that the records are present in.

Data is from:
iDigBio
Gbif
SFA (ASTC)
Mercer Arboretum
SHSU
Tropicos
Texas State Herbarium
UT Billie L. Turner Plant Resources Center
Individuals Matt White, Joe Liggio, Pauline Singleton, Eric Keith

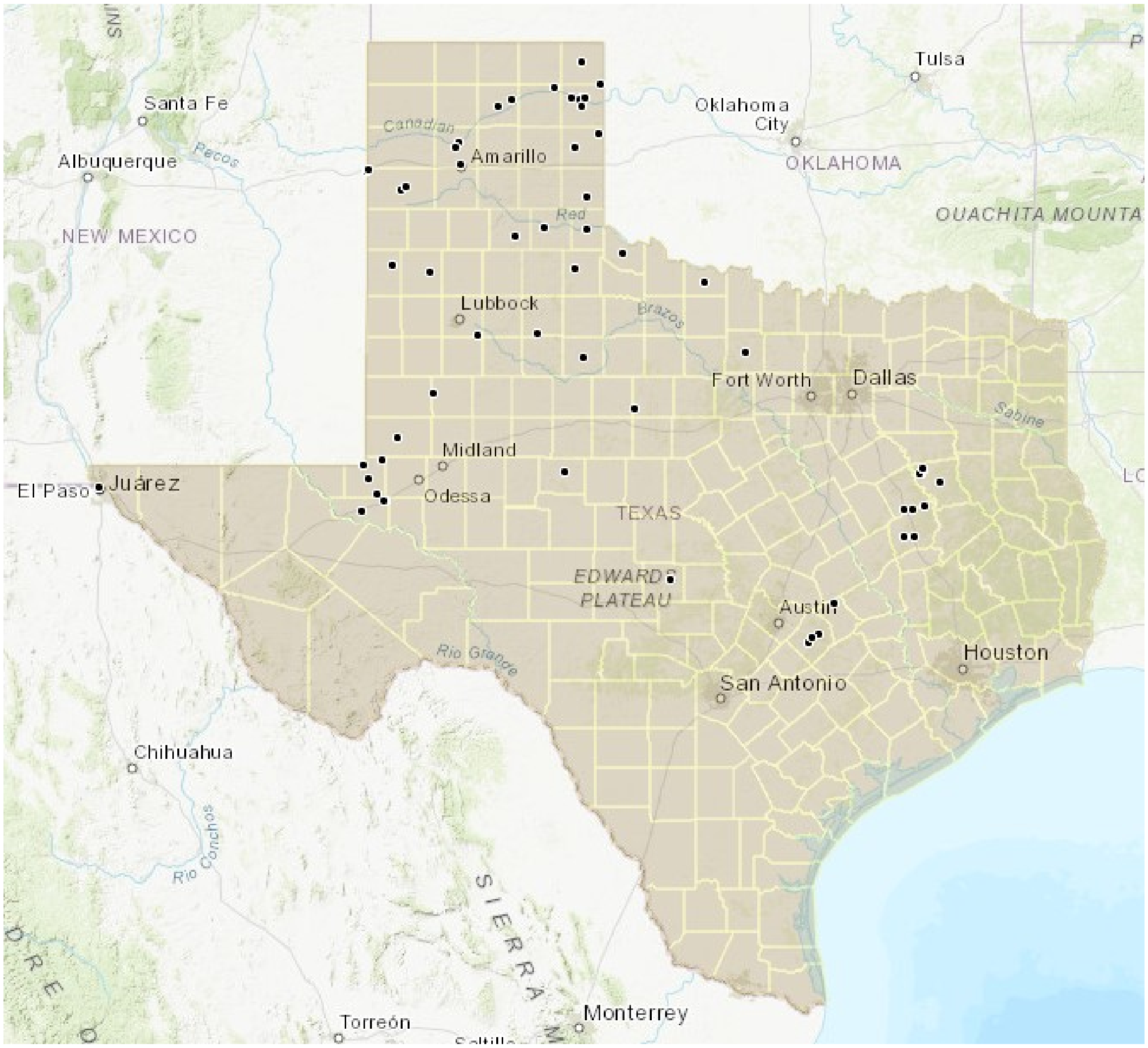
Asclepias amplexicaulis







Asclepias arenaria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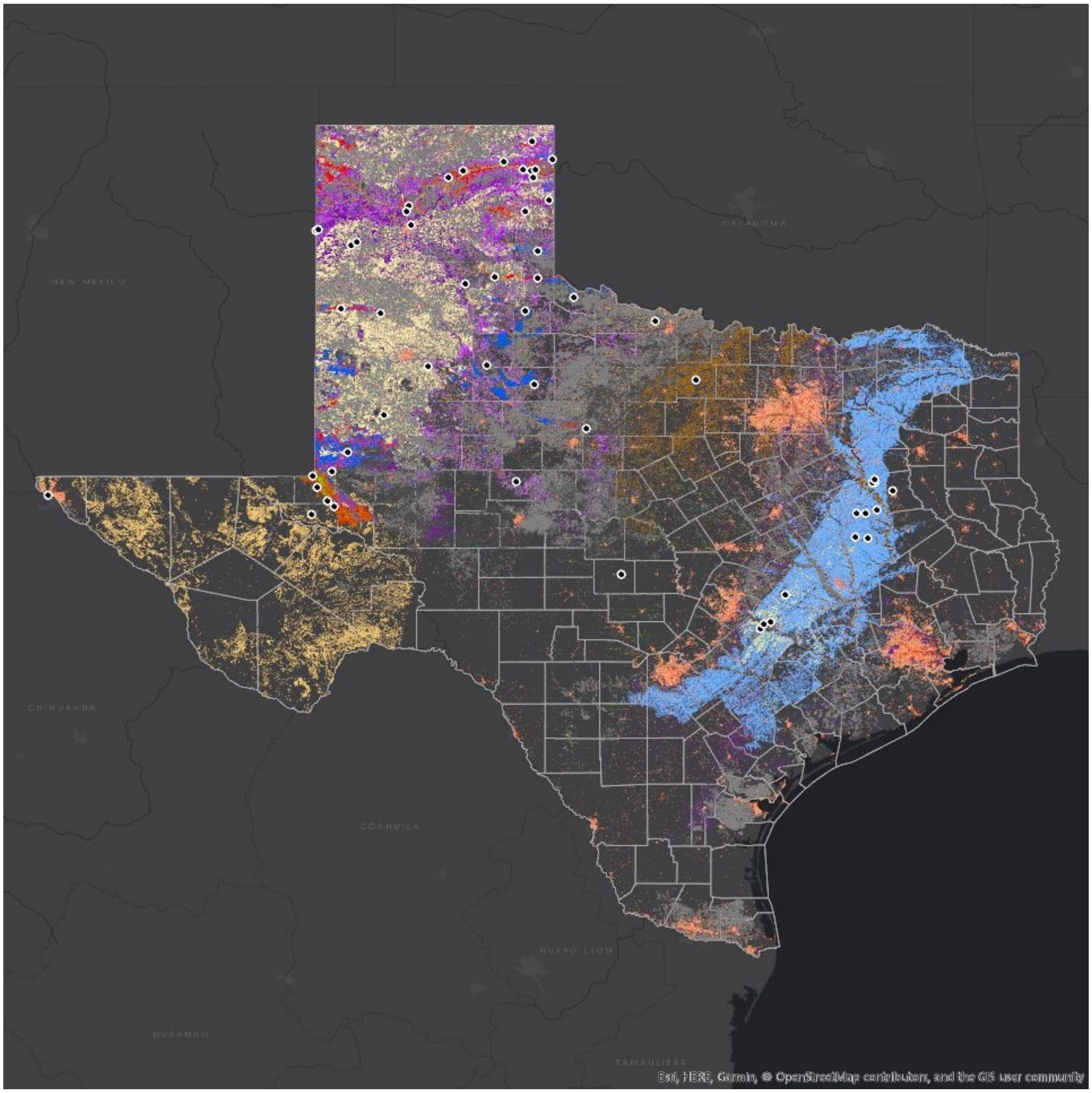
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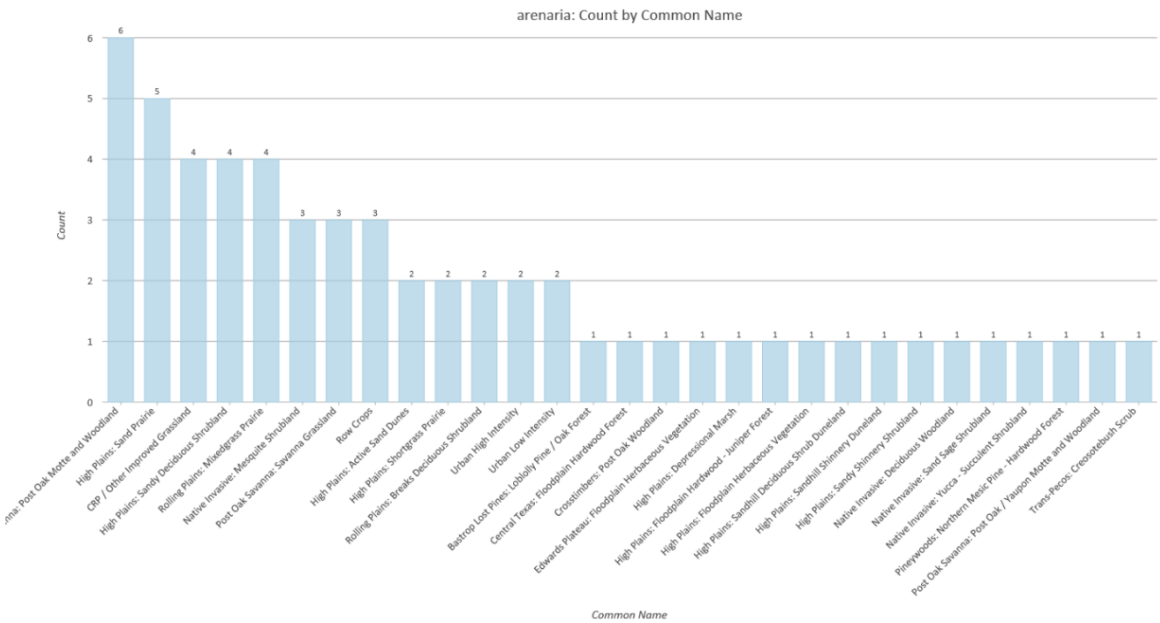
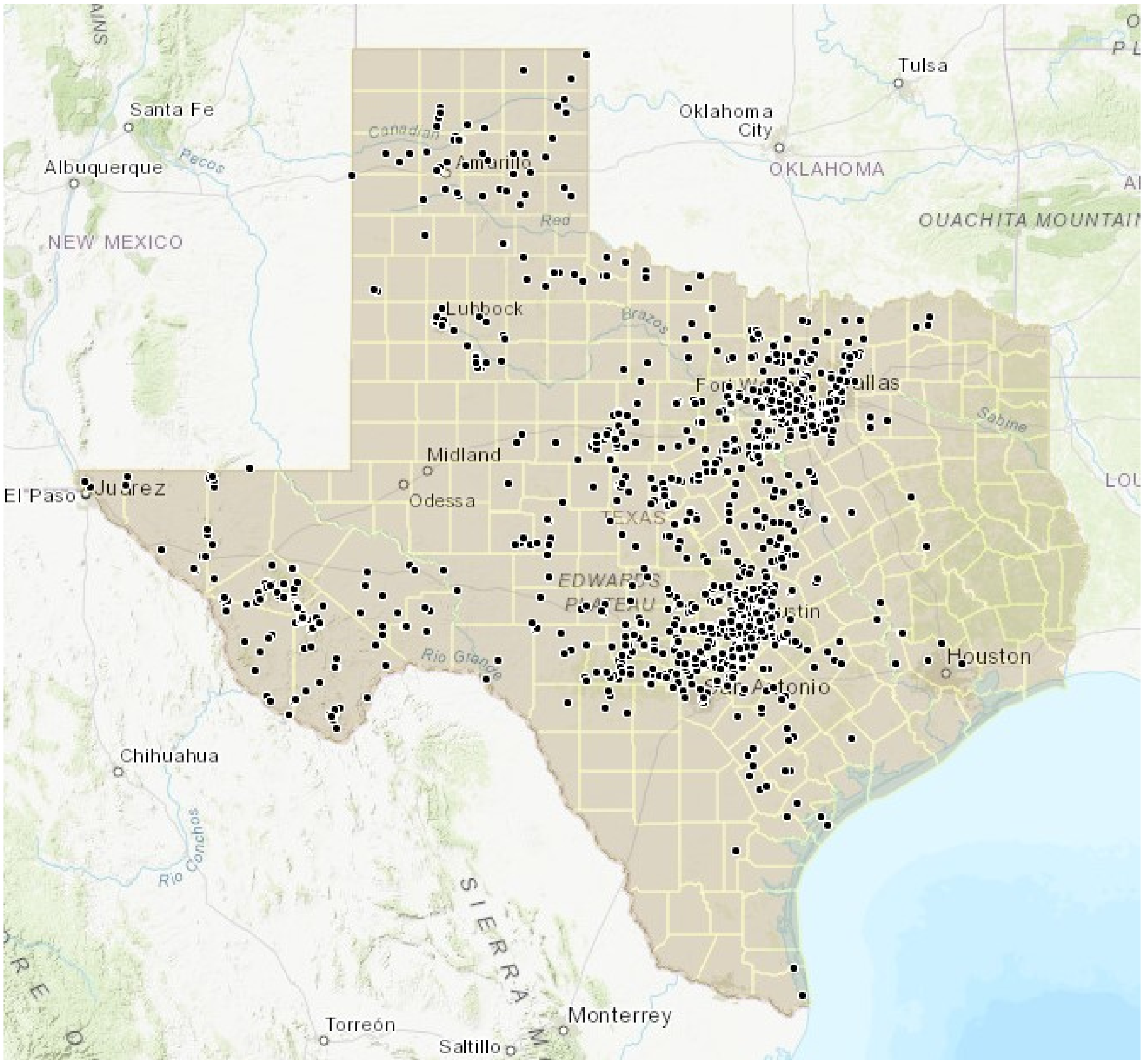


Asclepias asperula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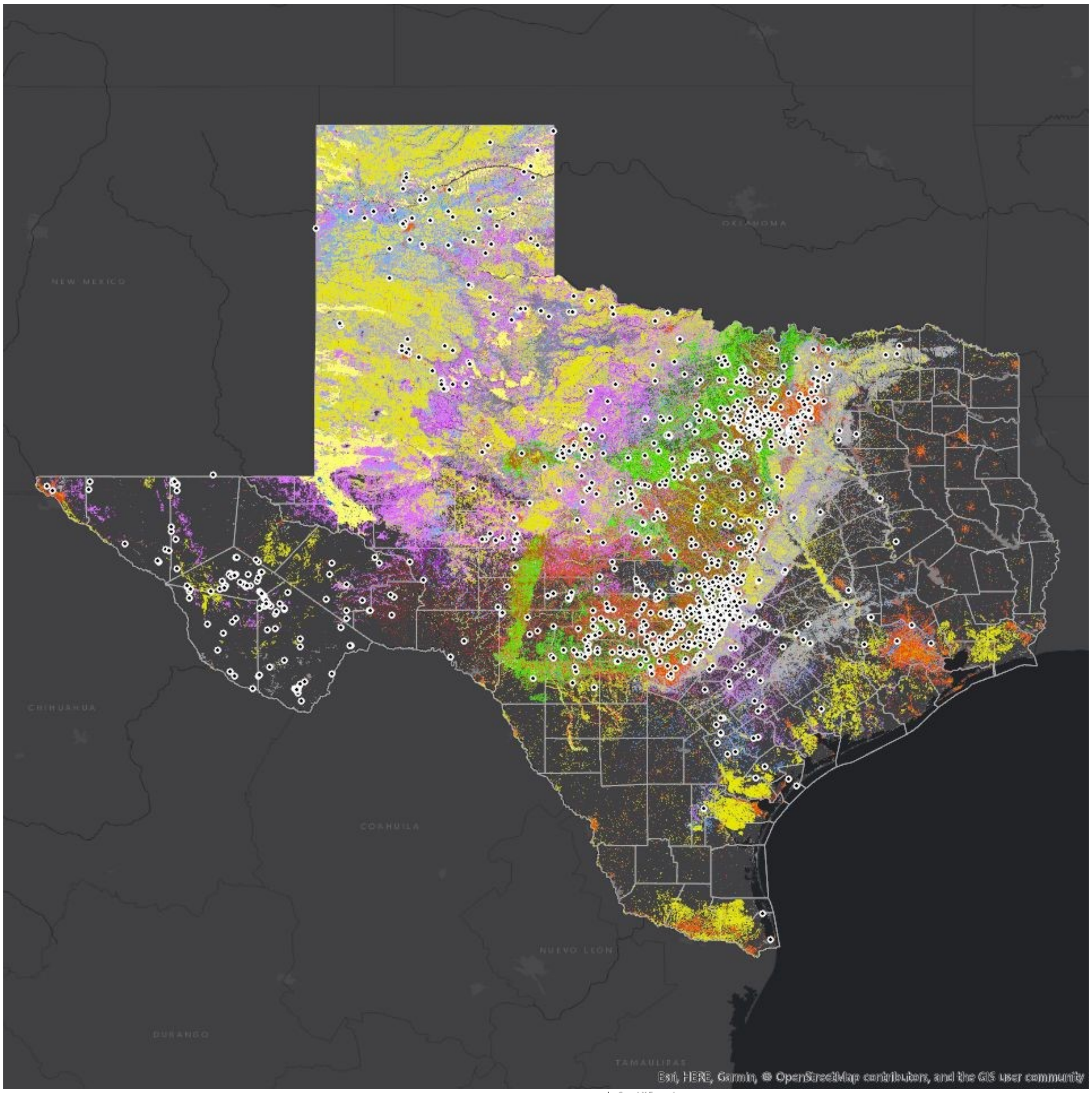
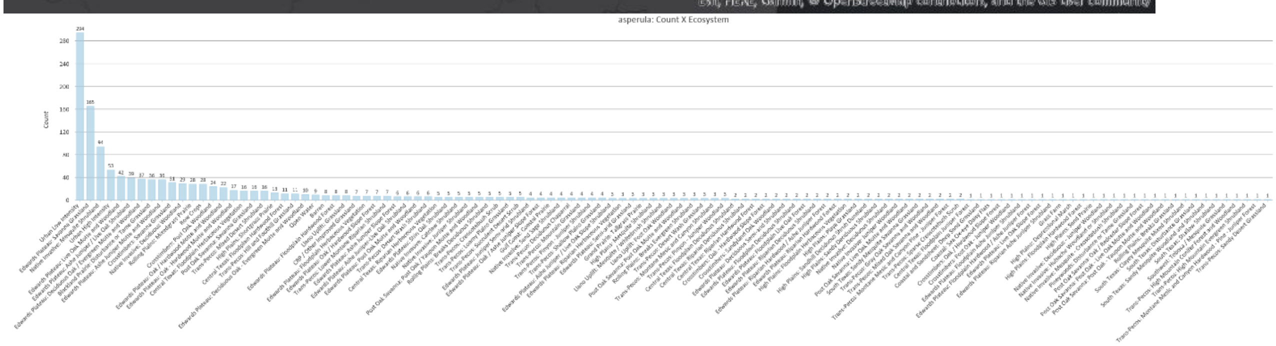
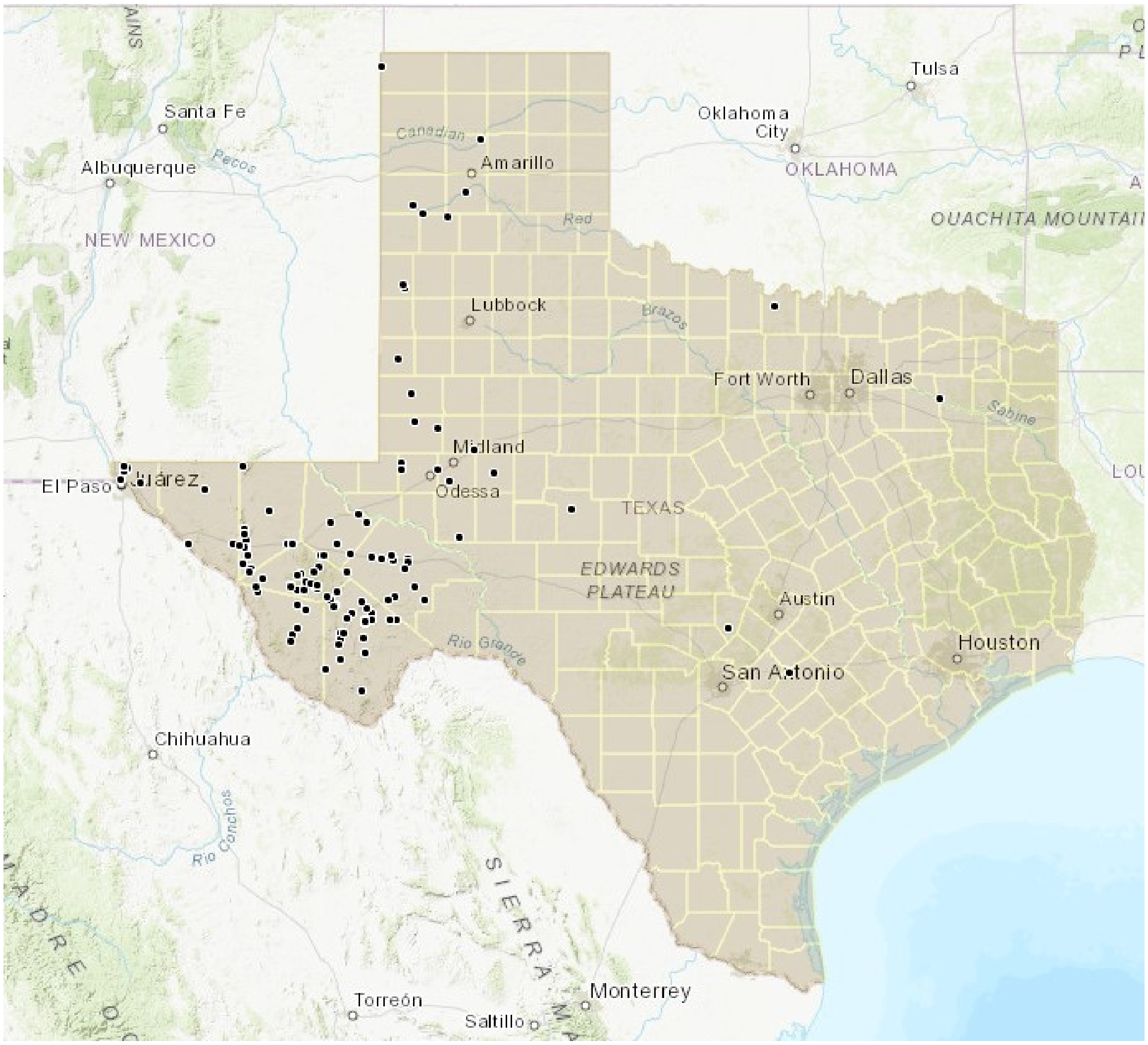




Asclepias brachystephana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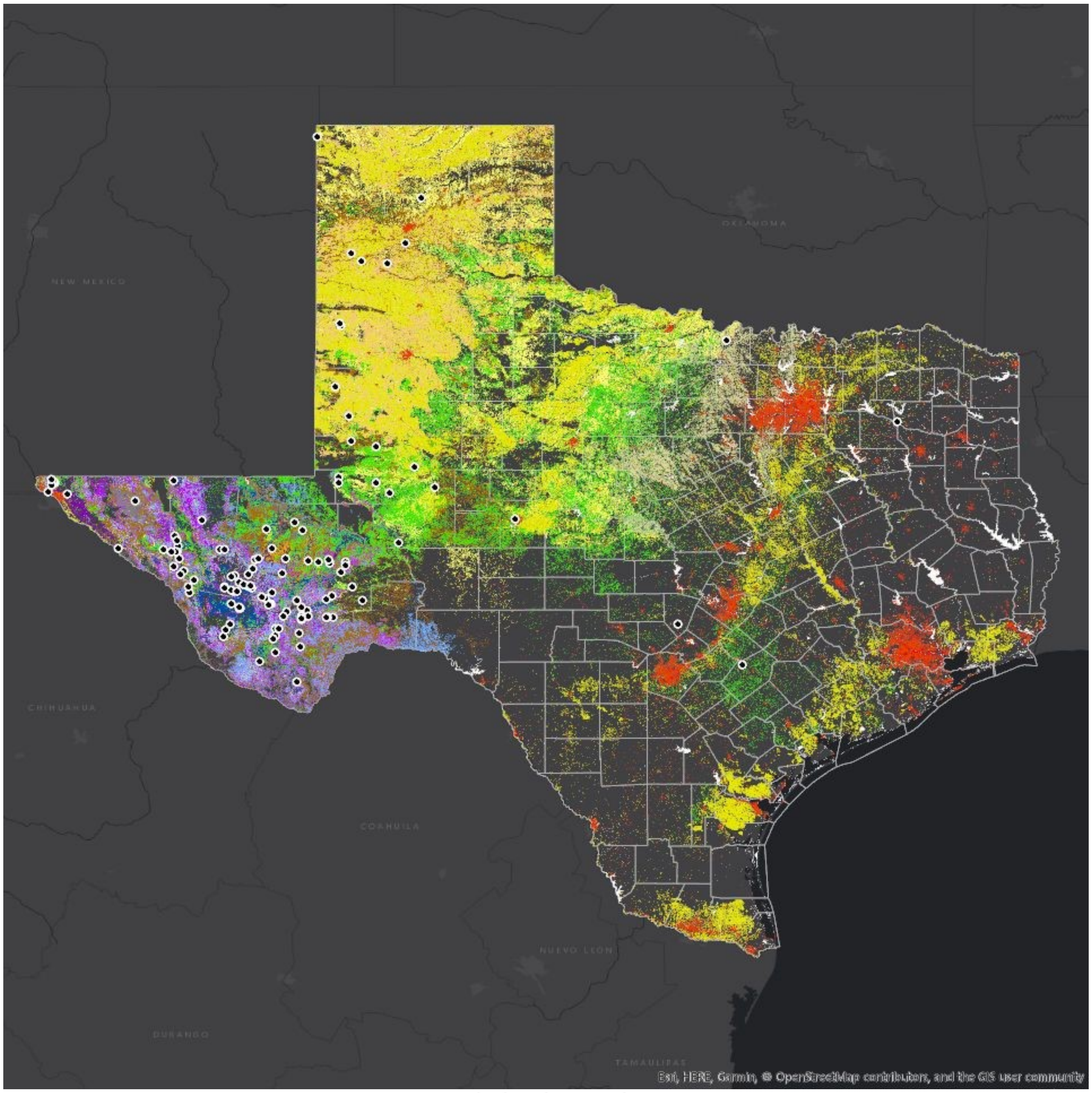
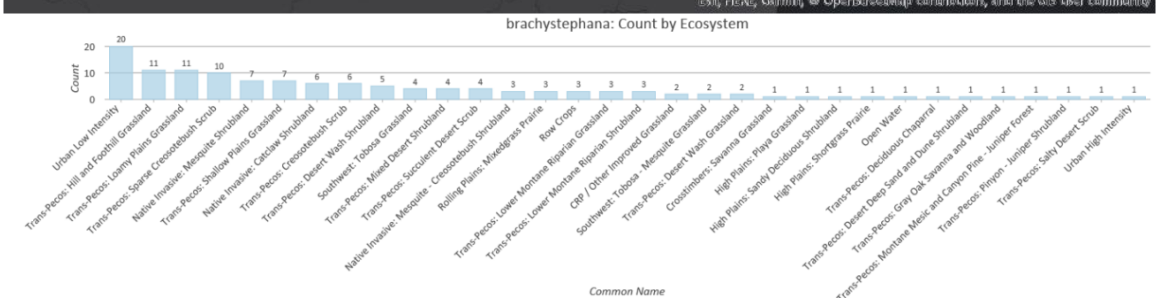
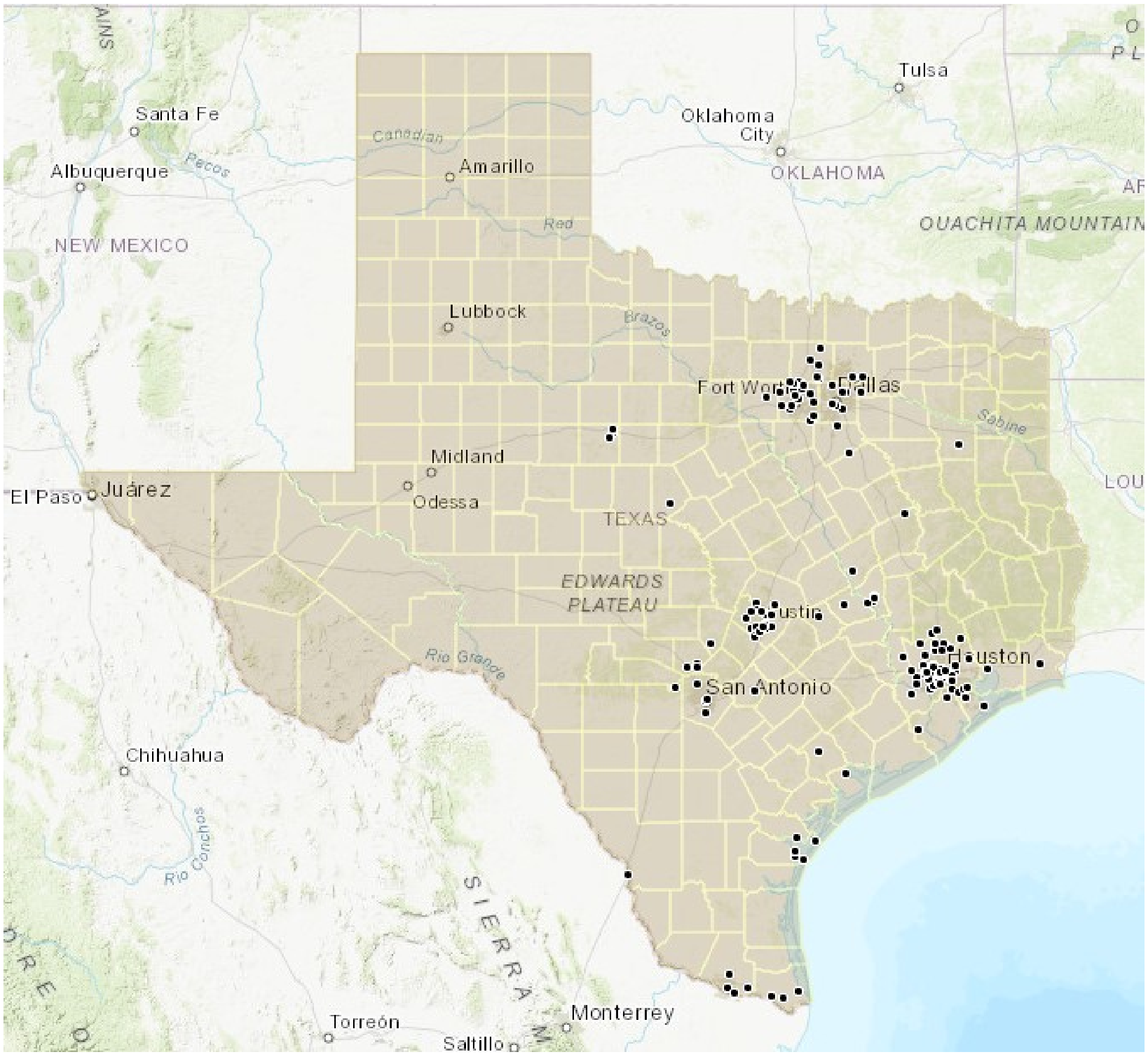




Asclepias curassavica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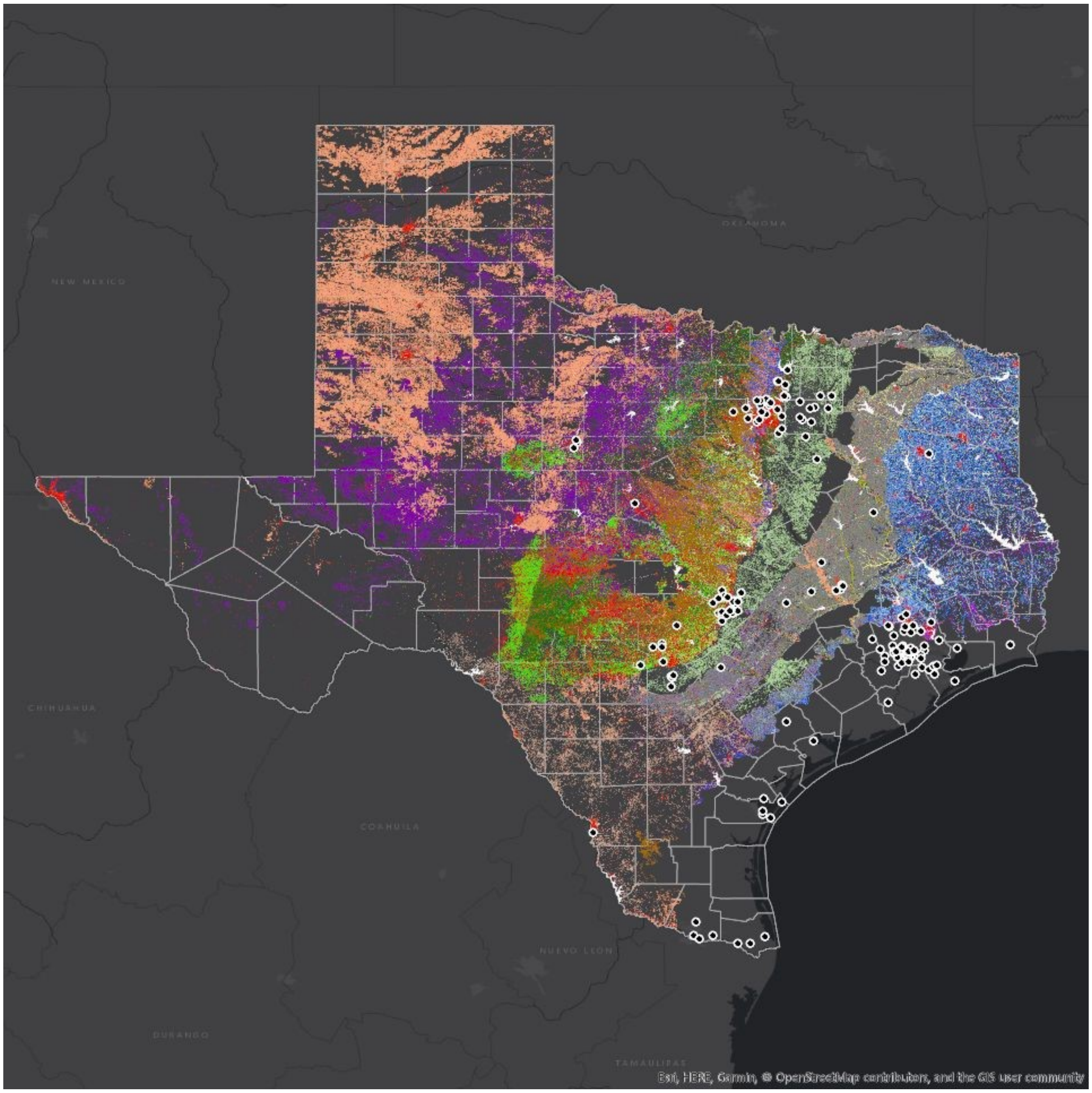
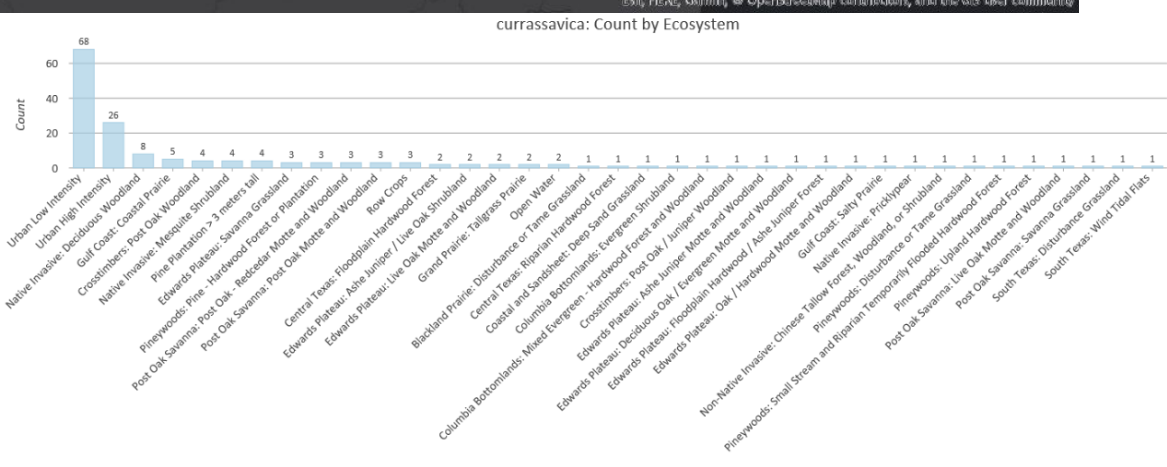
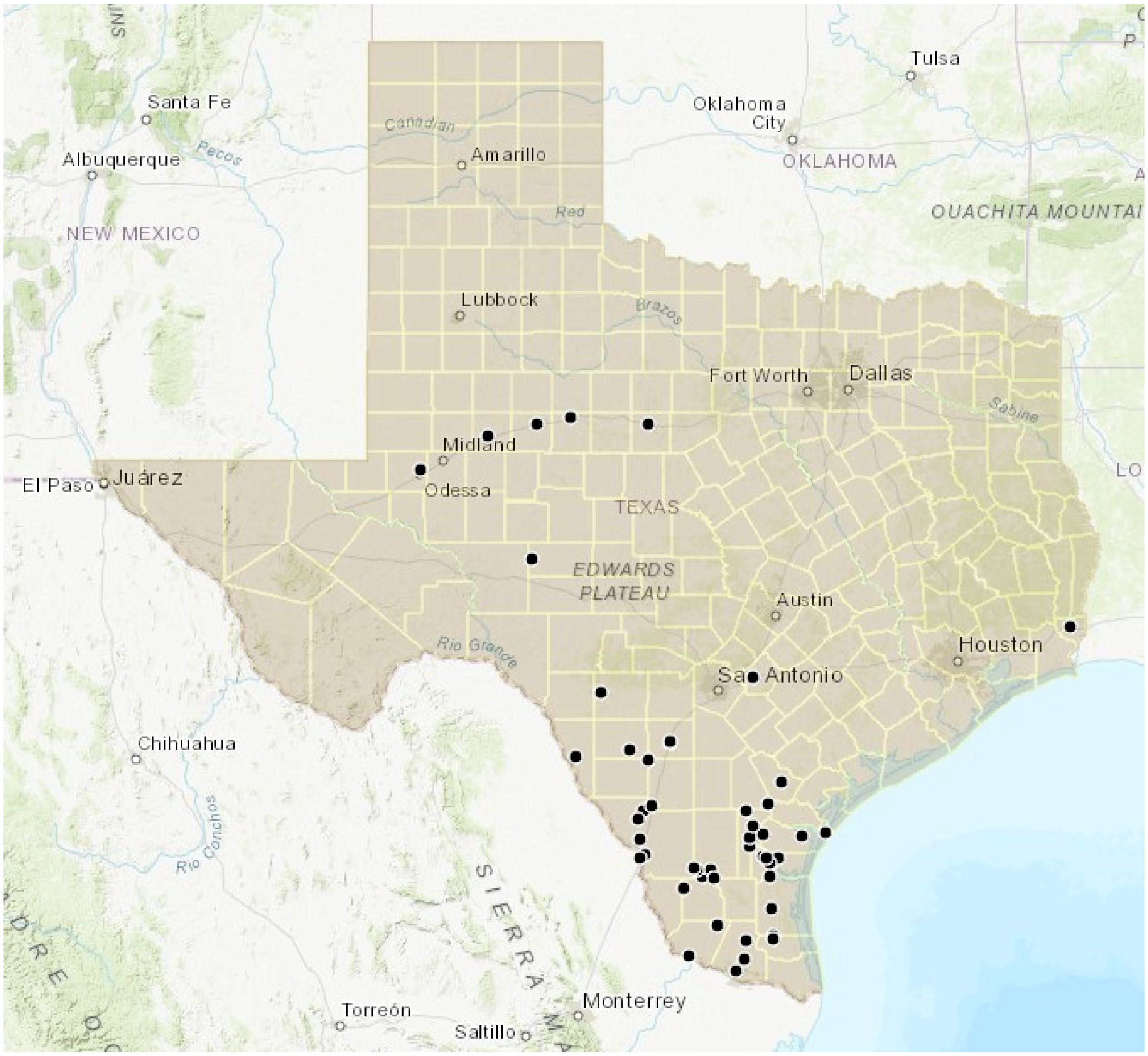




Asclepias emoryi



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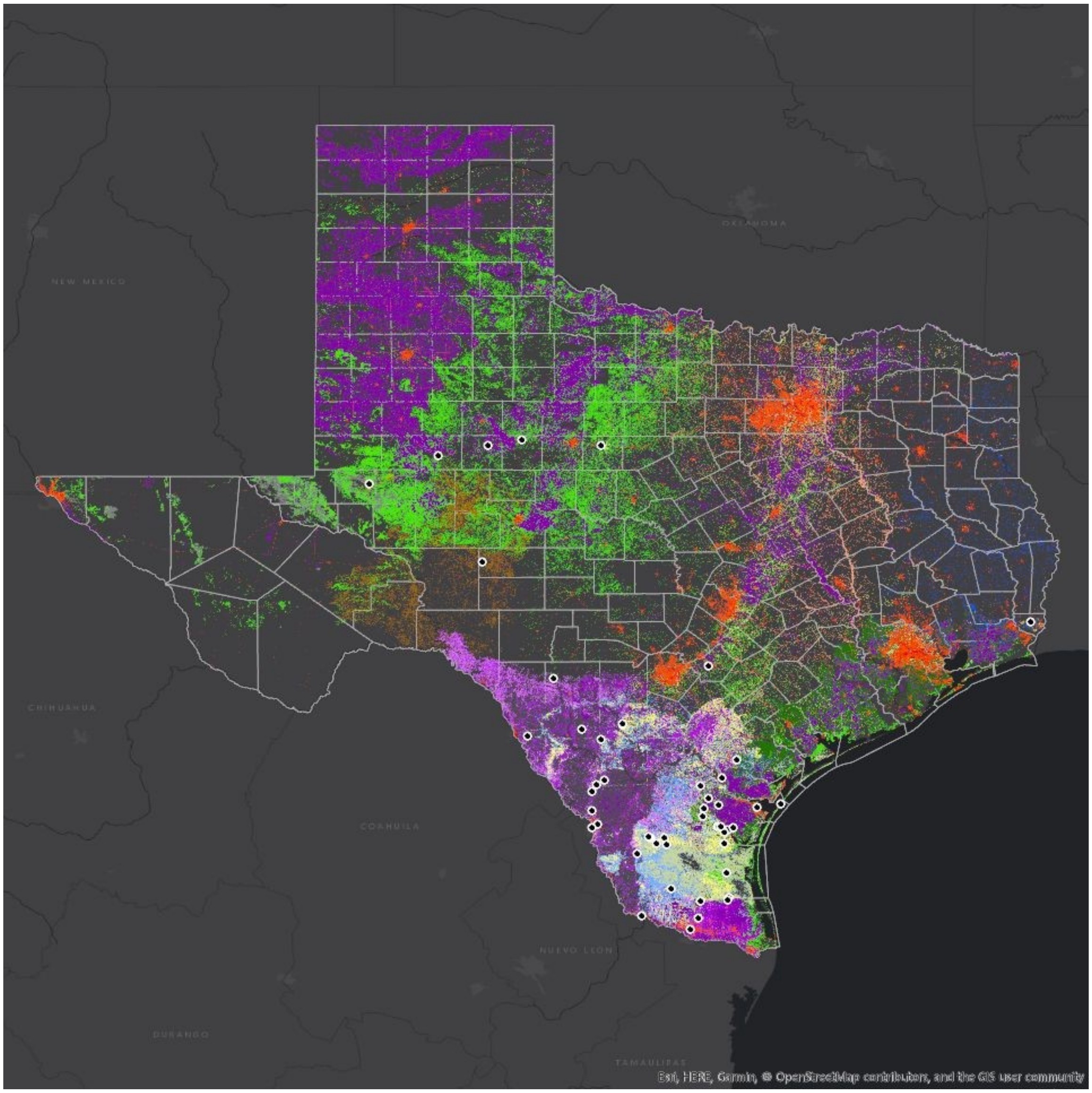
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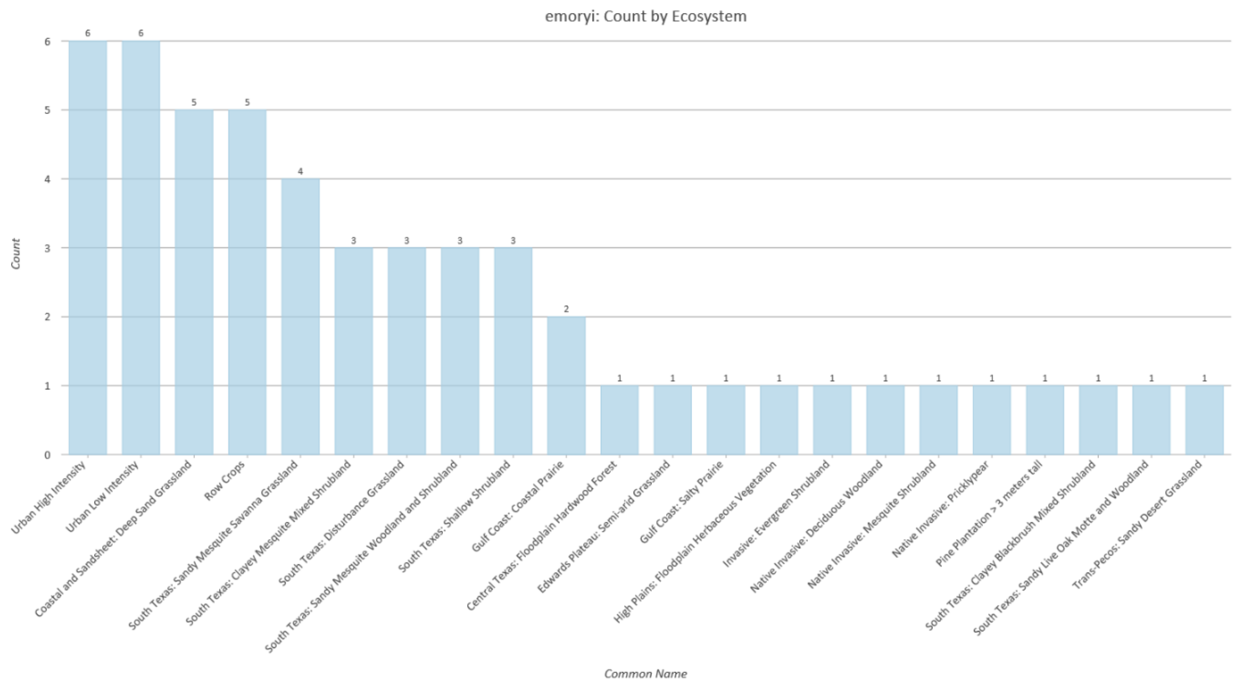
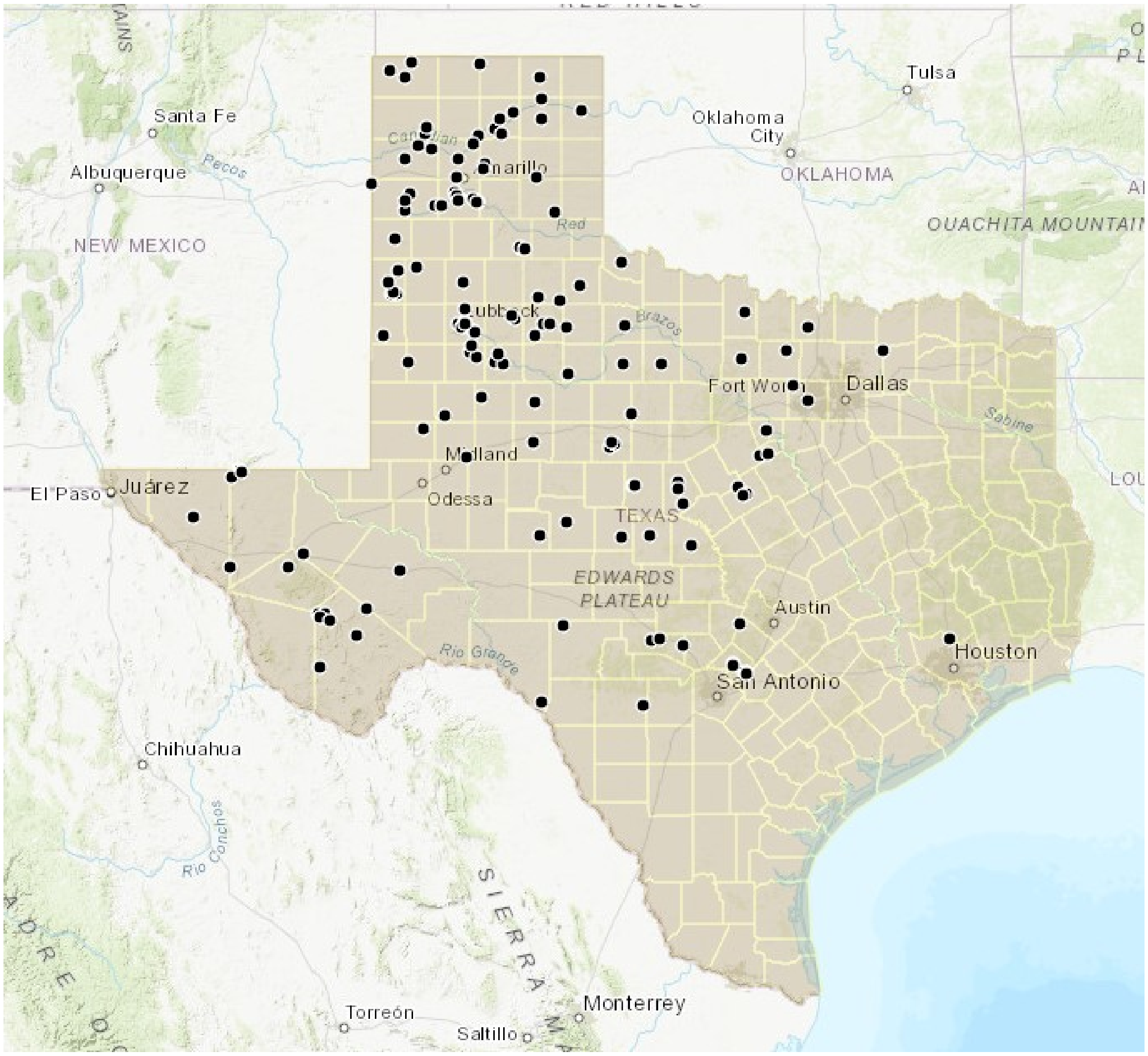


Asclepias engelmaniana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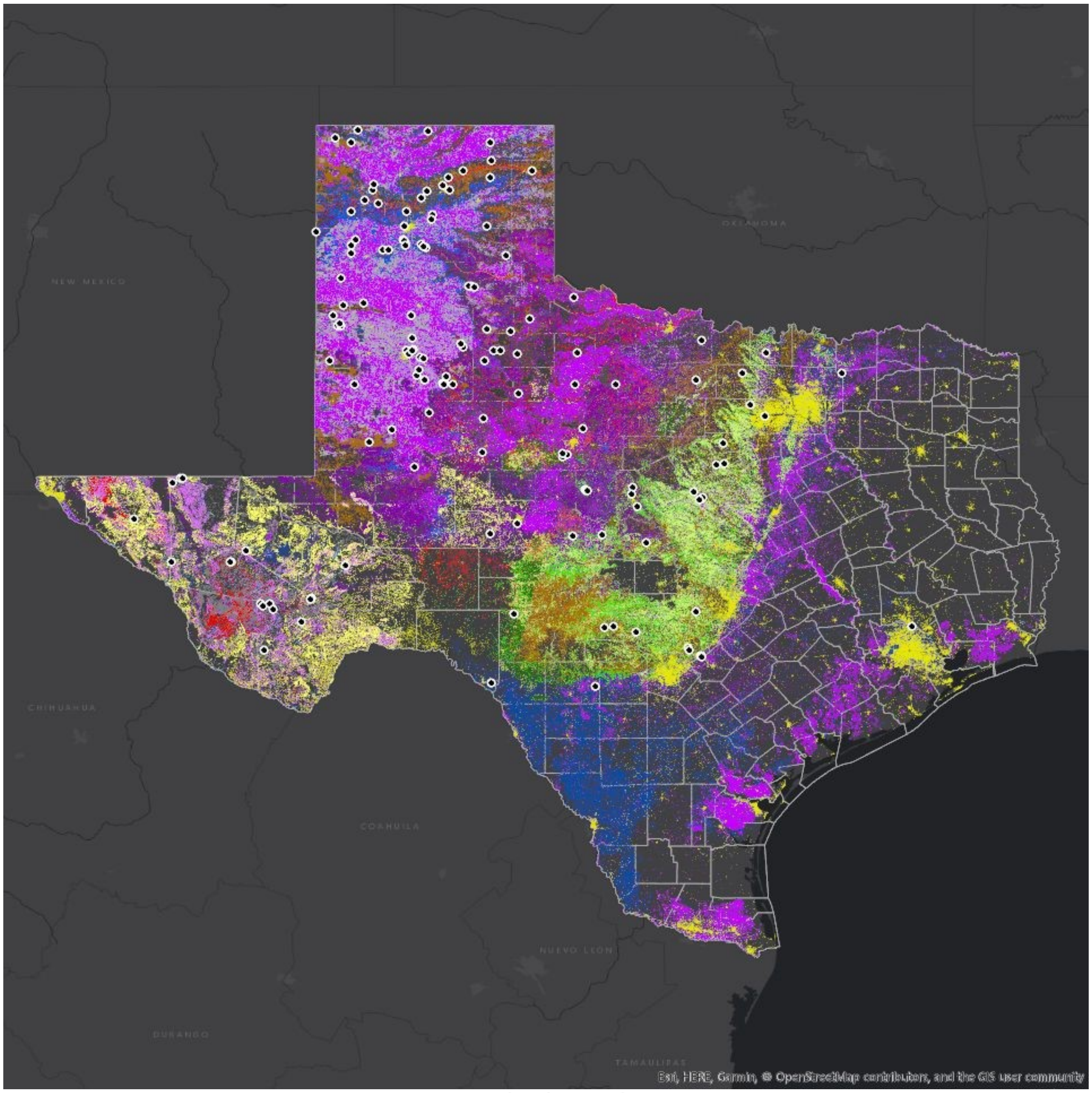
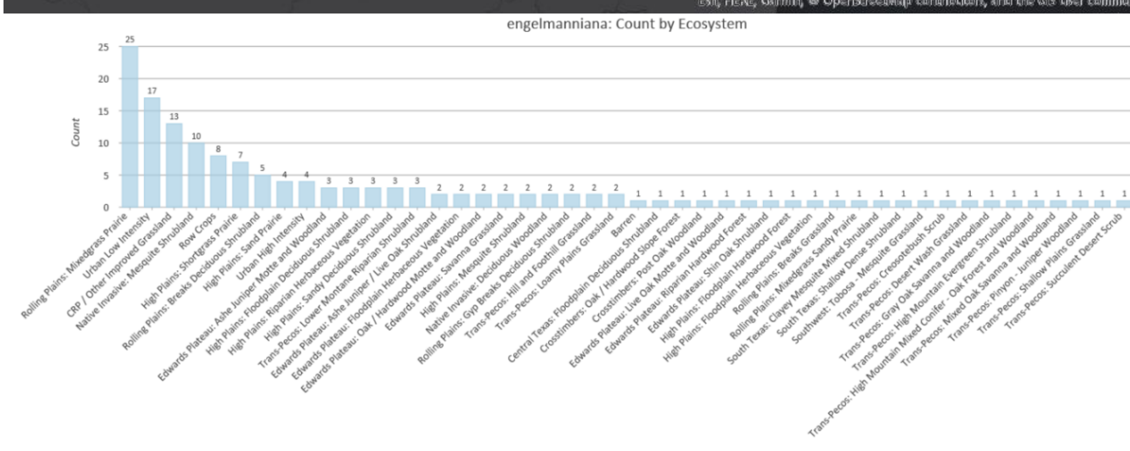
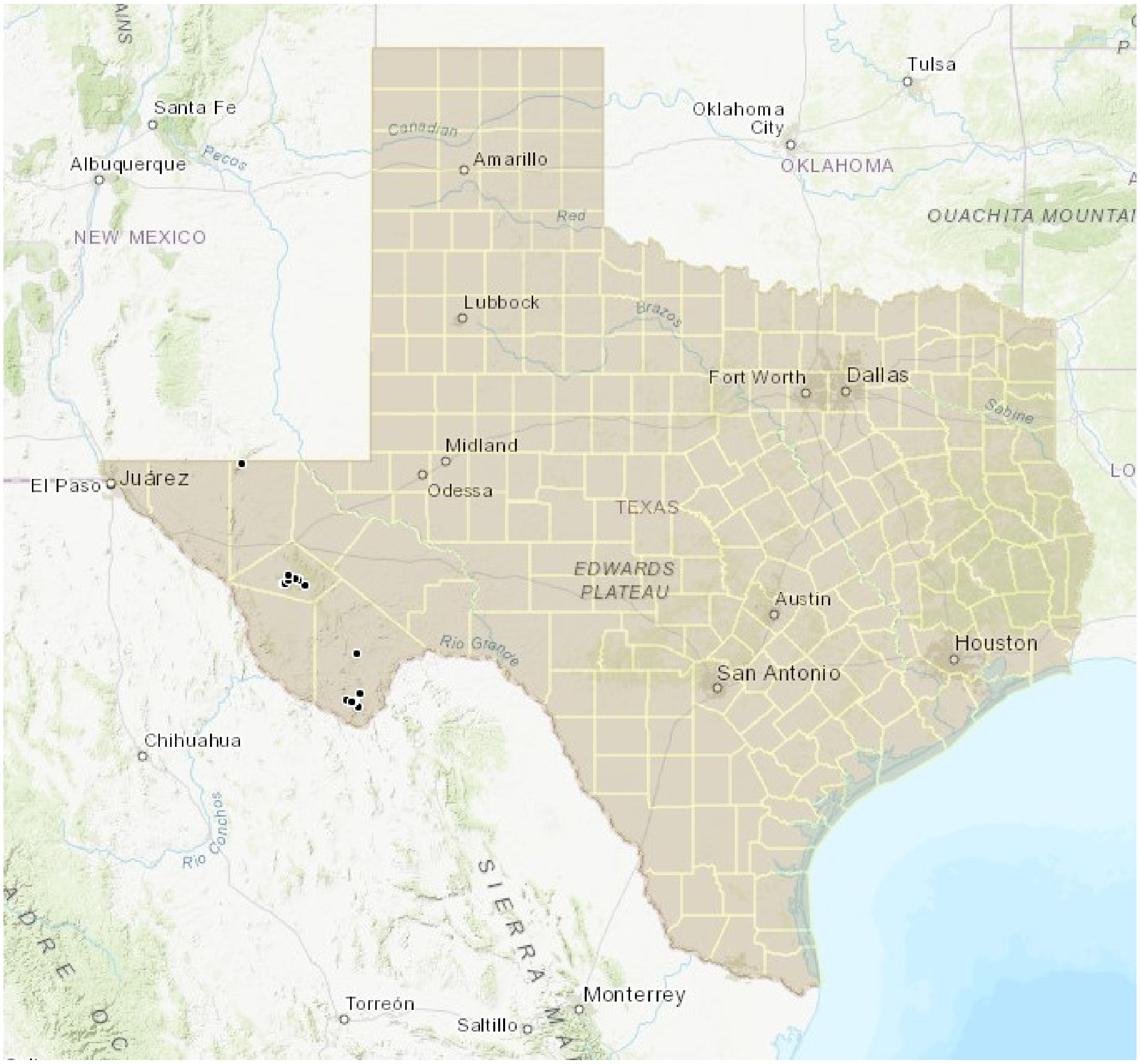




Asclepias glaucescens



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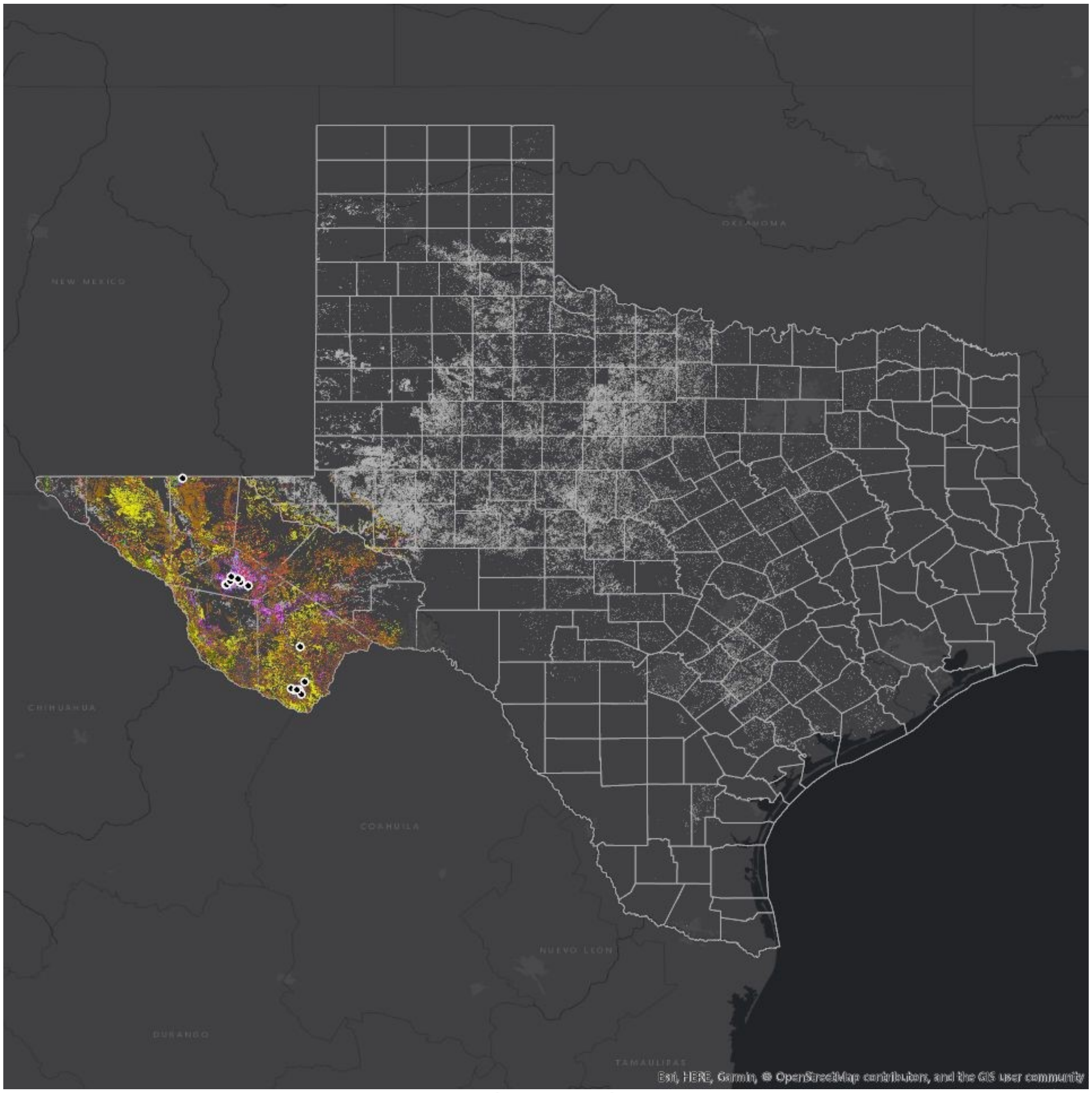
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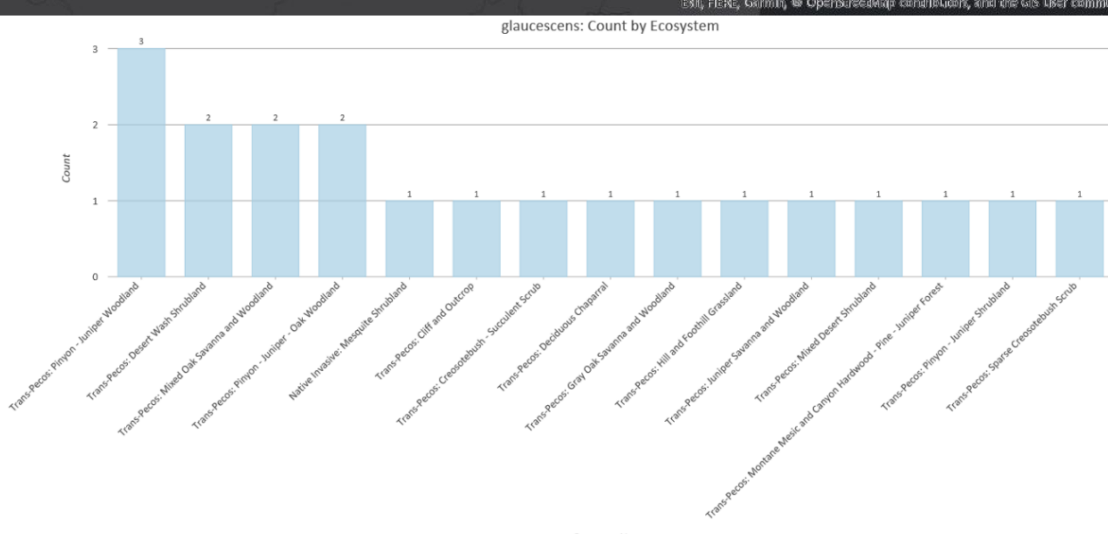
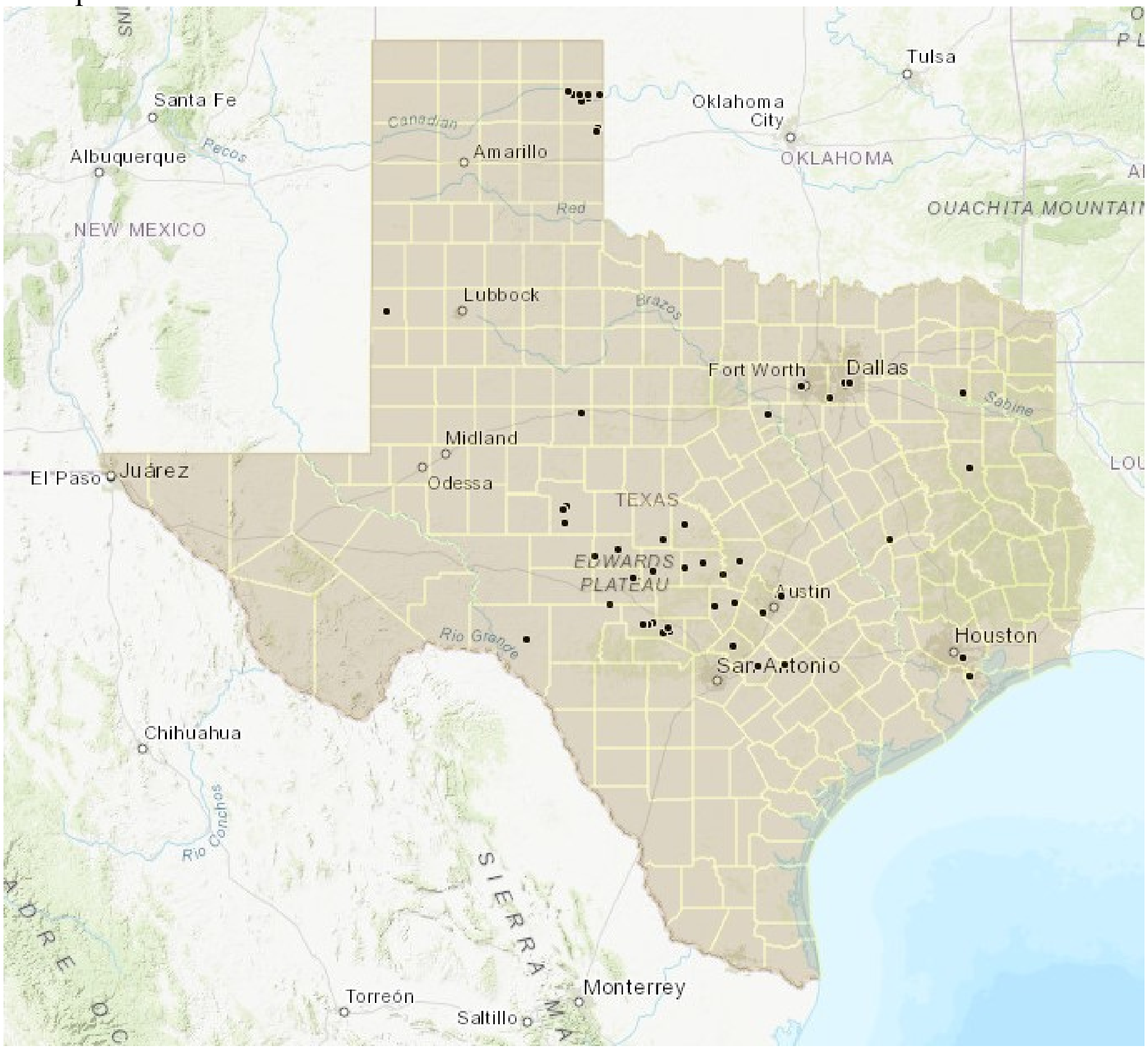
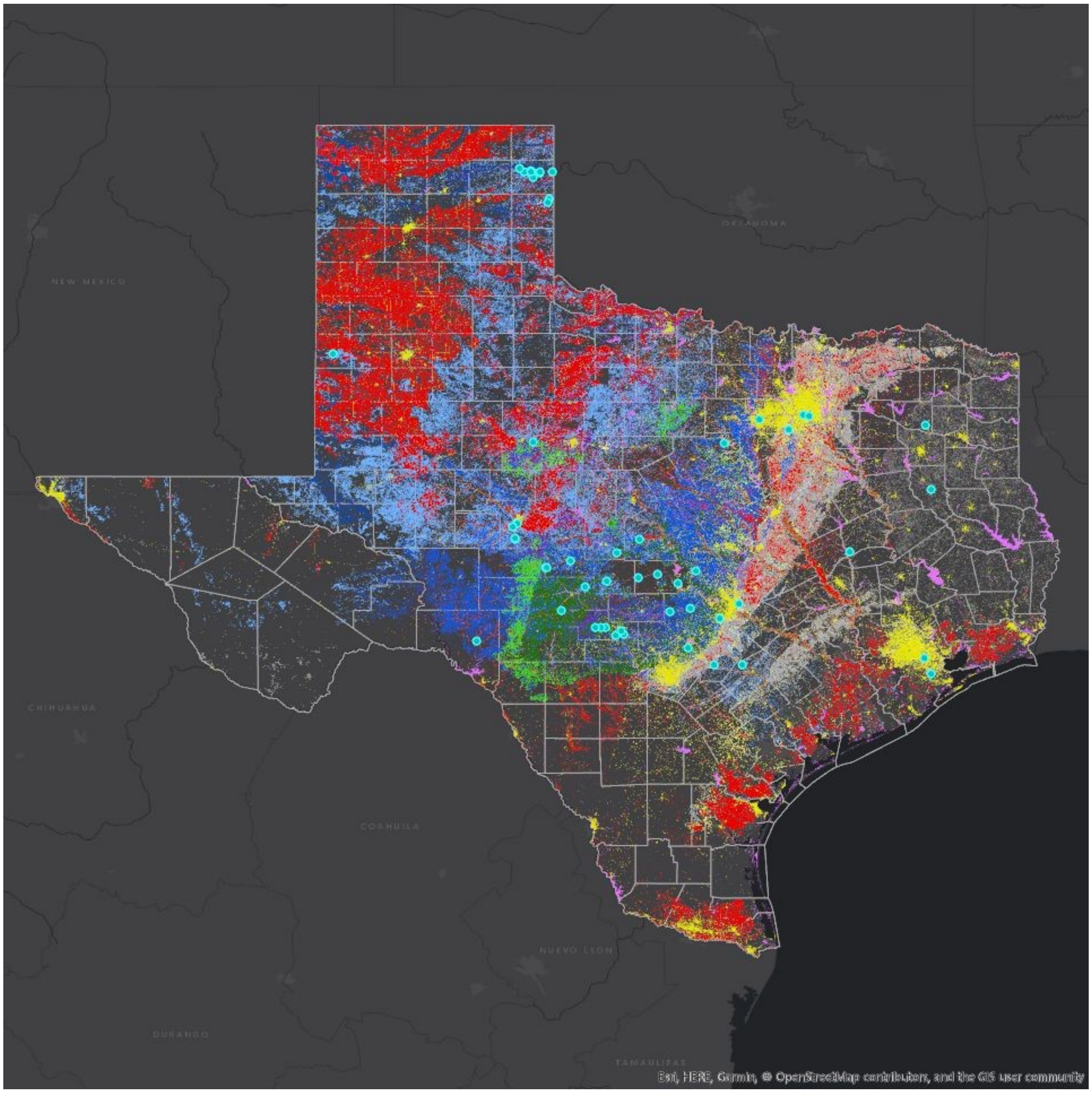

glaucescens: Count by Ecosystem
Count
Common Name
Trans-Pecos: Pinyon - Juniper Woodland
Trans-Pecos: Desert Wash Shrubland
Trans-Pecos: Mixed Oak Savanna and Woodland
Trans-Pecos: Pinyon - Juniper - Oak Woodland
Native Invasive: Mesquite Shrubland
Trans-Pecos: Cliff and Outcrop
Trans-Pecos: Creosotebush - Succulent Scrub
Trans-Pecos: Deciduous Chaparral
Trans-Pecos: Gray Oak Savanna and Woodland
Trans-Pecos: Hill and Foothill Grassland
Trans-Pecos: Juniper Savanna and Woodland
Trans-Pecos: Mixed Desert Shrubland
Trans-Pecos: Montane Mesic and Canyon Hardwood - Pine - Juniper Forest
Trans-Pecos: Pinyon - Juniper Shrubland
Trans-Pecos: Sparse Creosotebush Scrub

Asclepias incarnata





Asclepias involucrata

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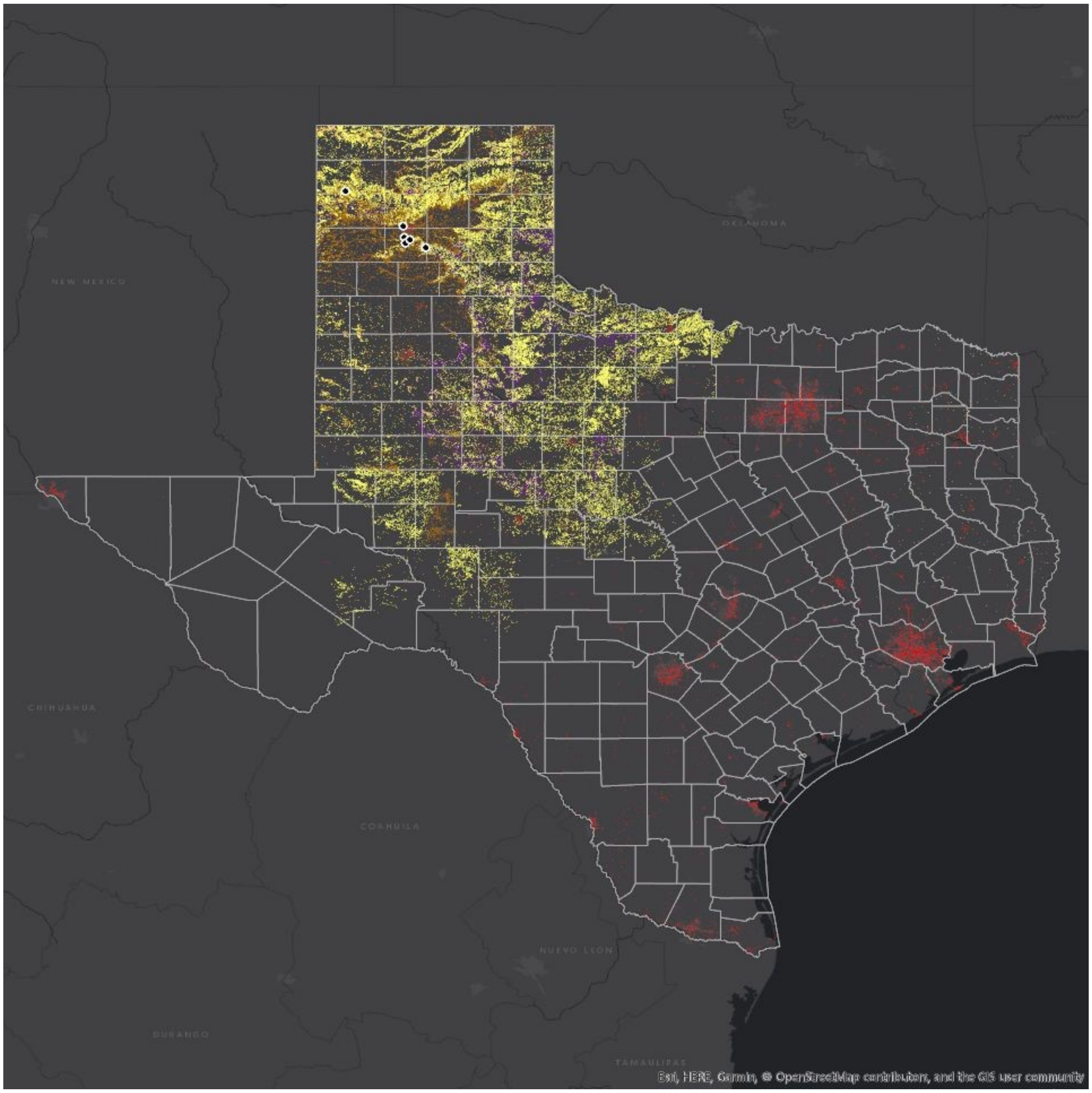
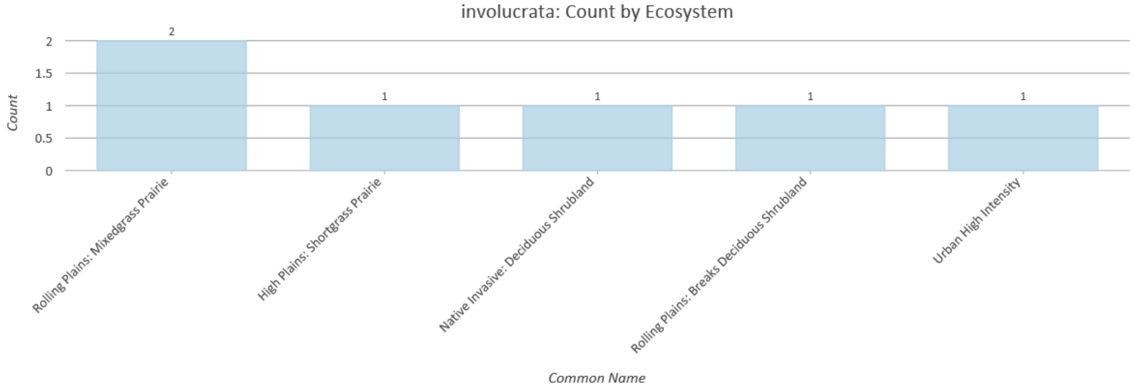
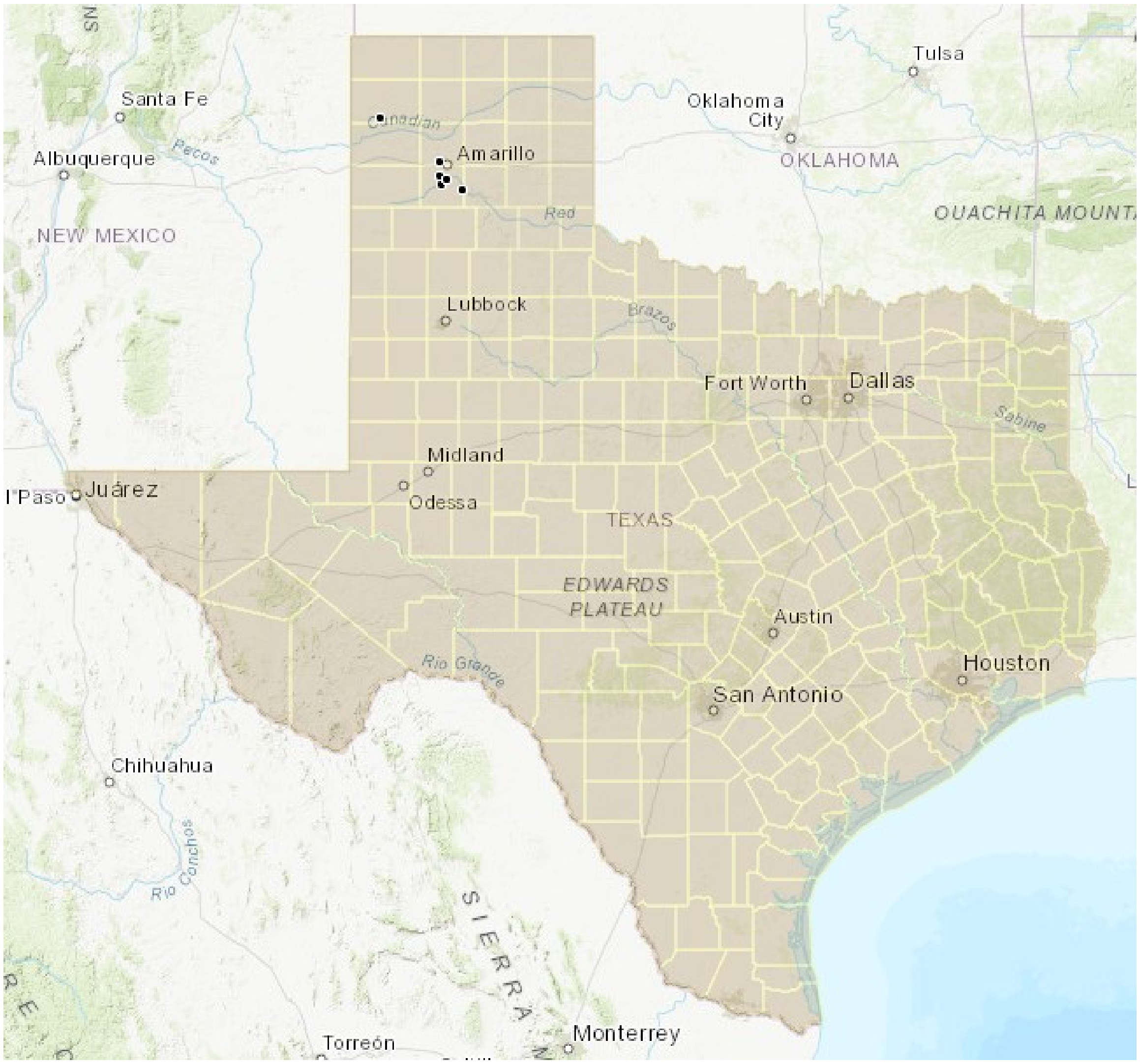


involucrata: Count by Ecosystem



Asclepias lanceolata



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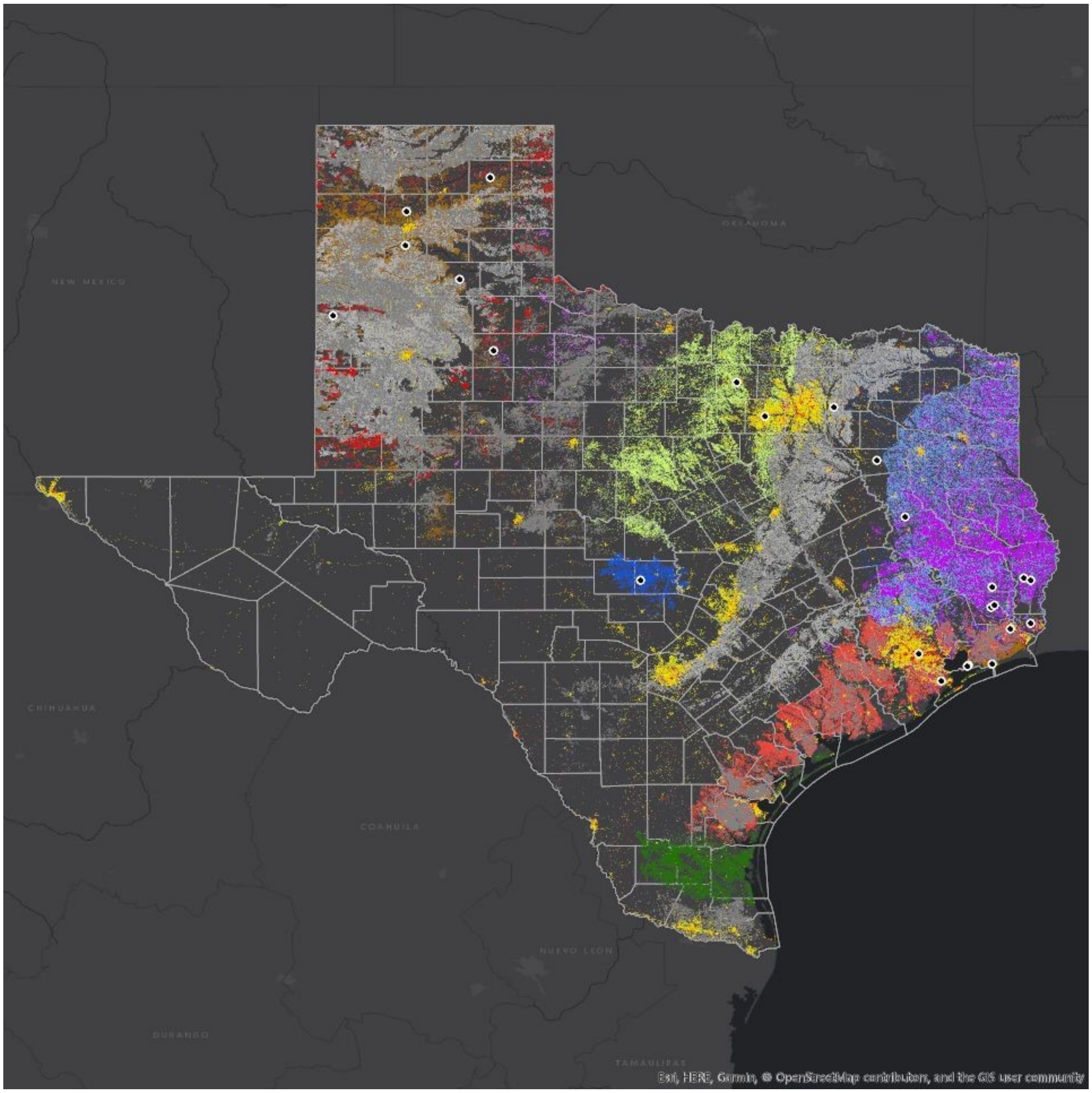
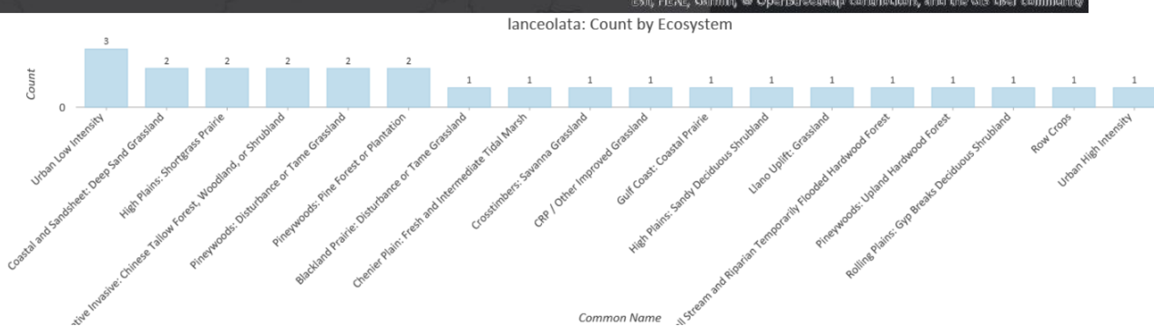
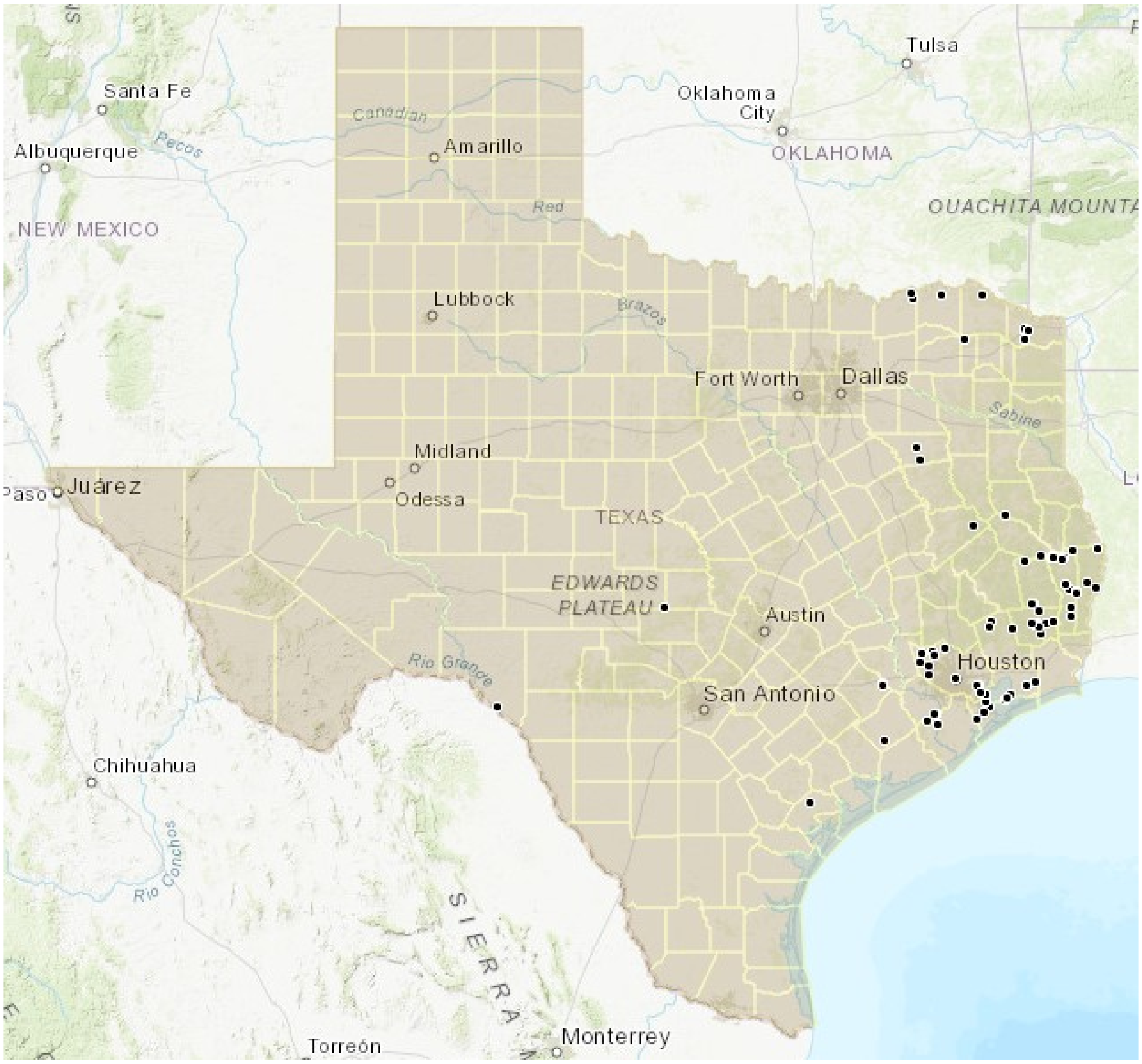




Asclepias latifolia



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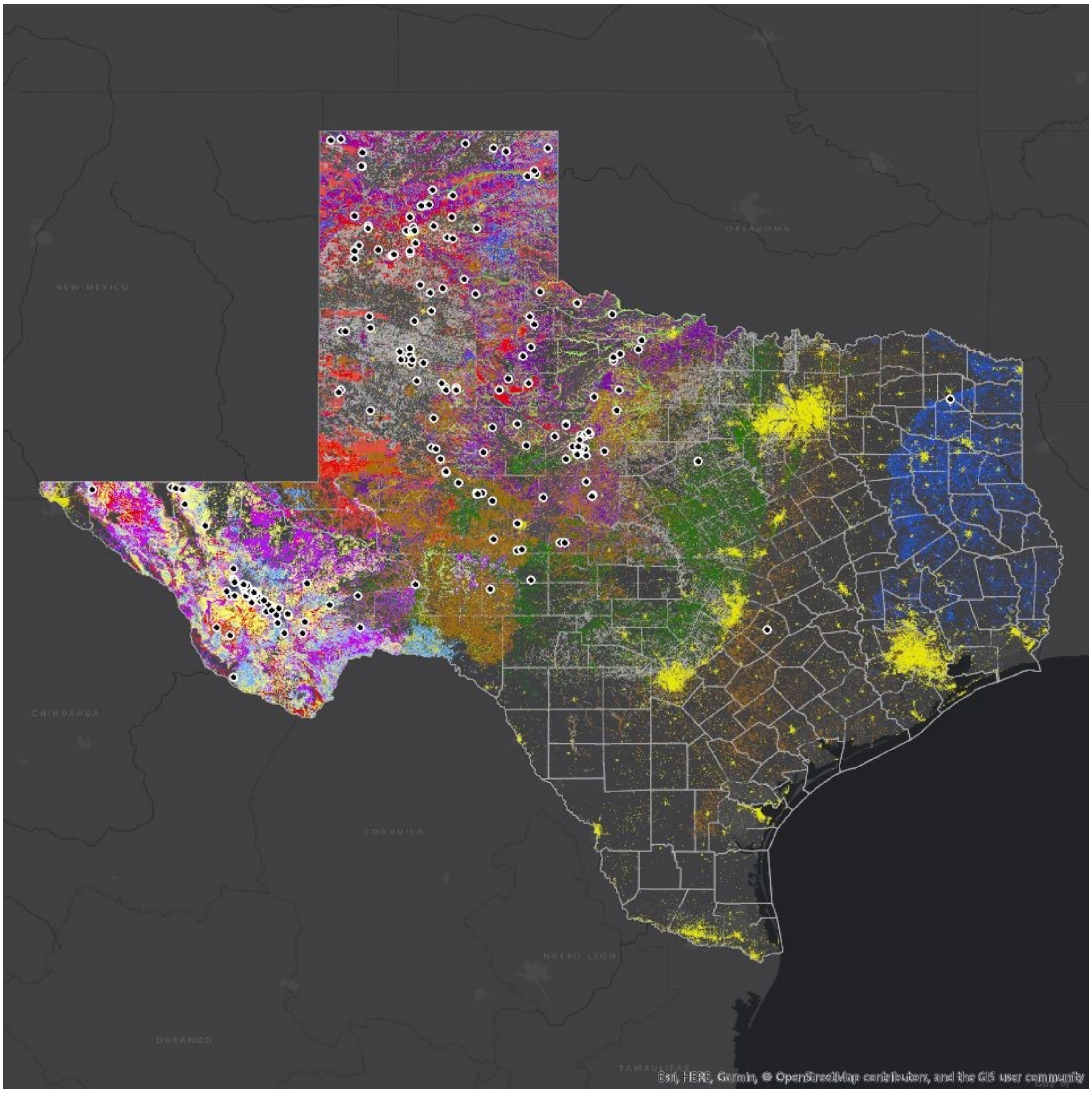
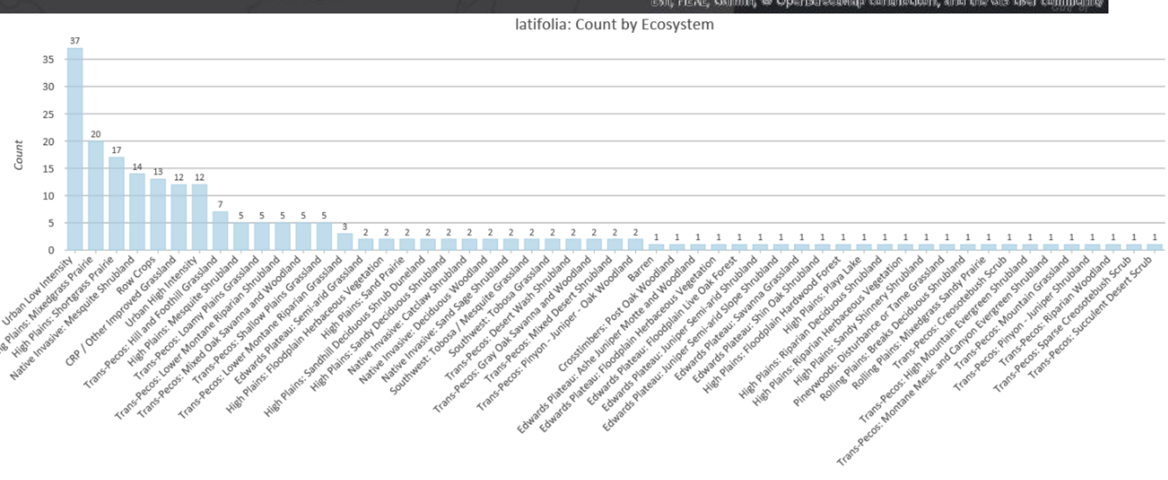
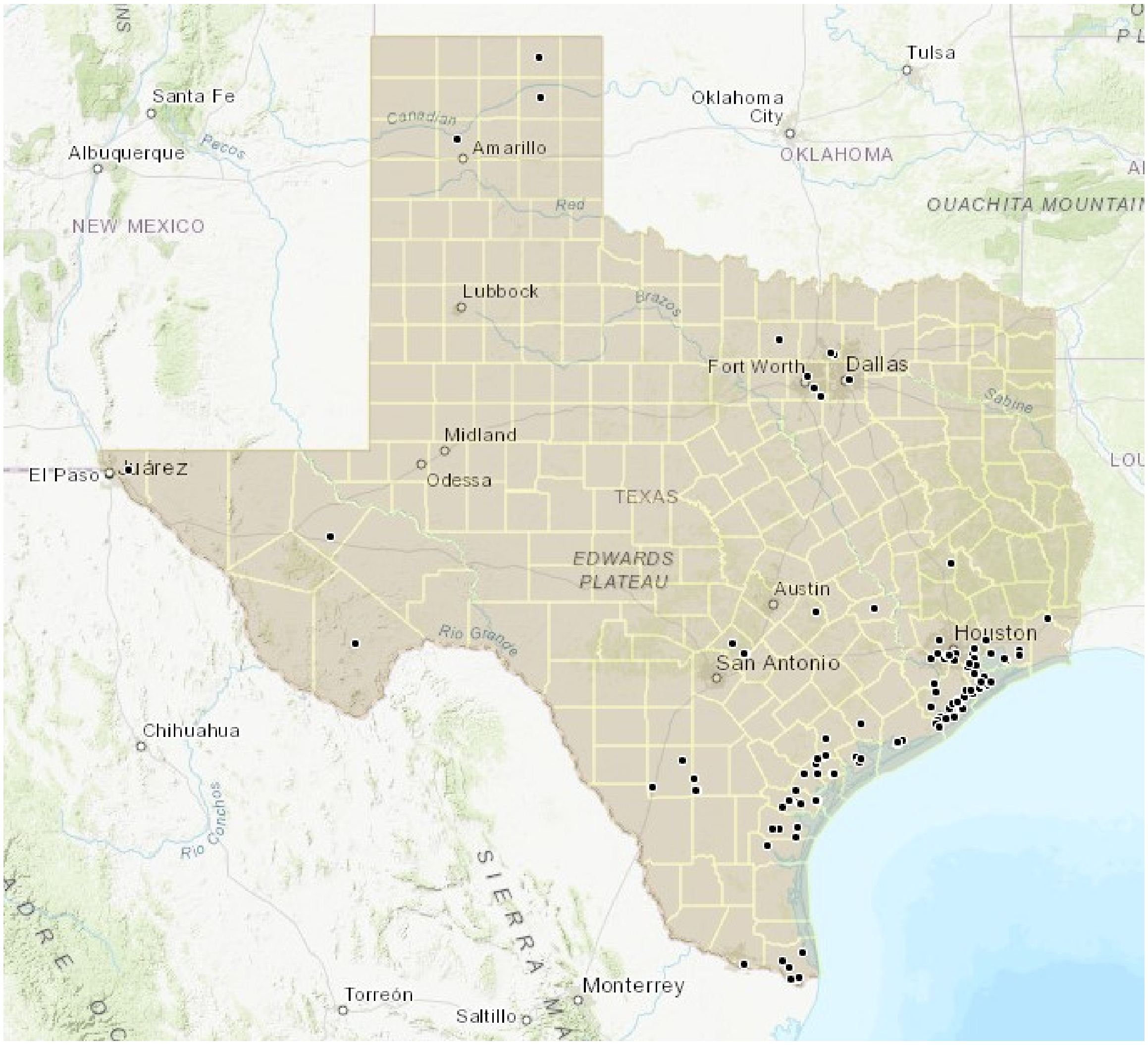




Asclepias linearis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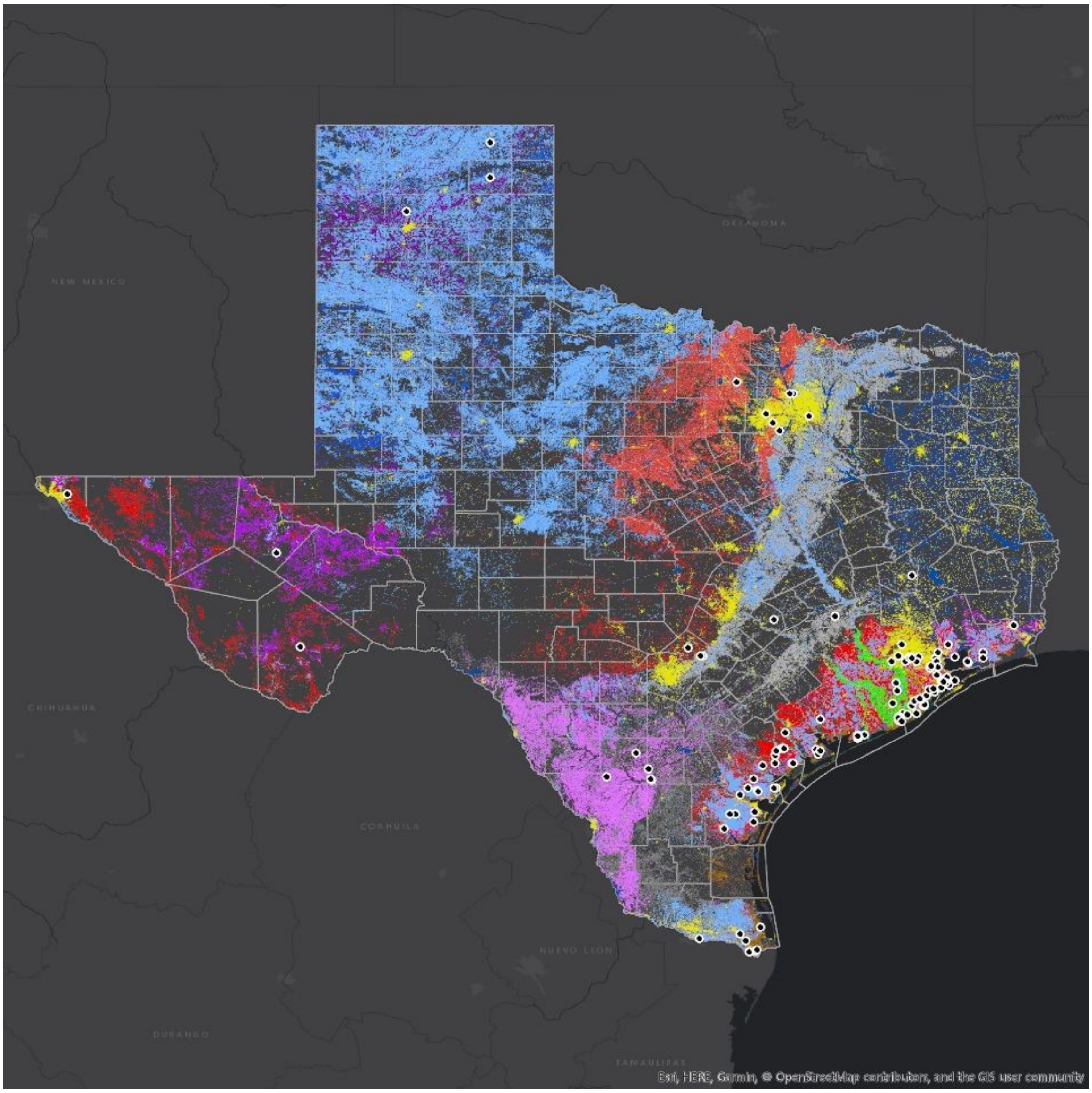
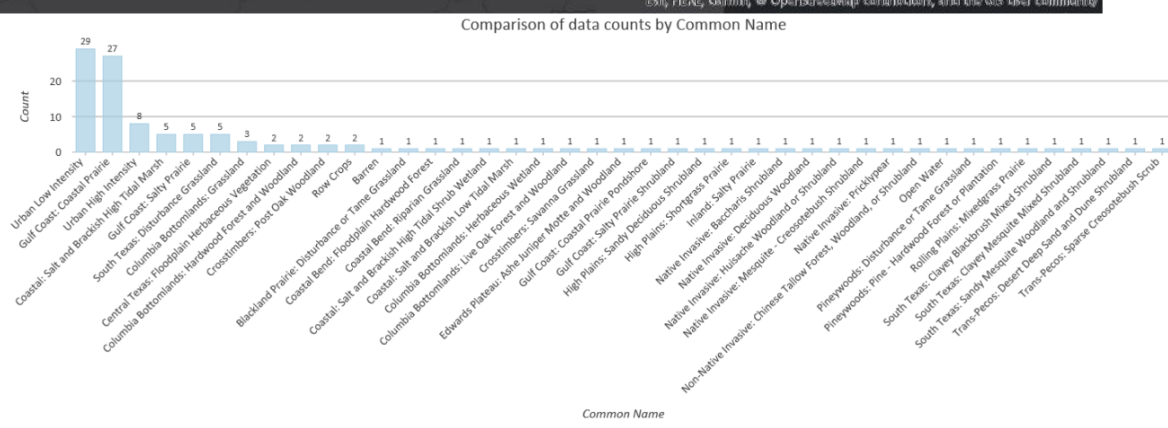
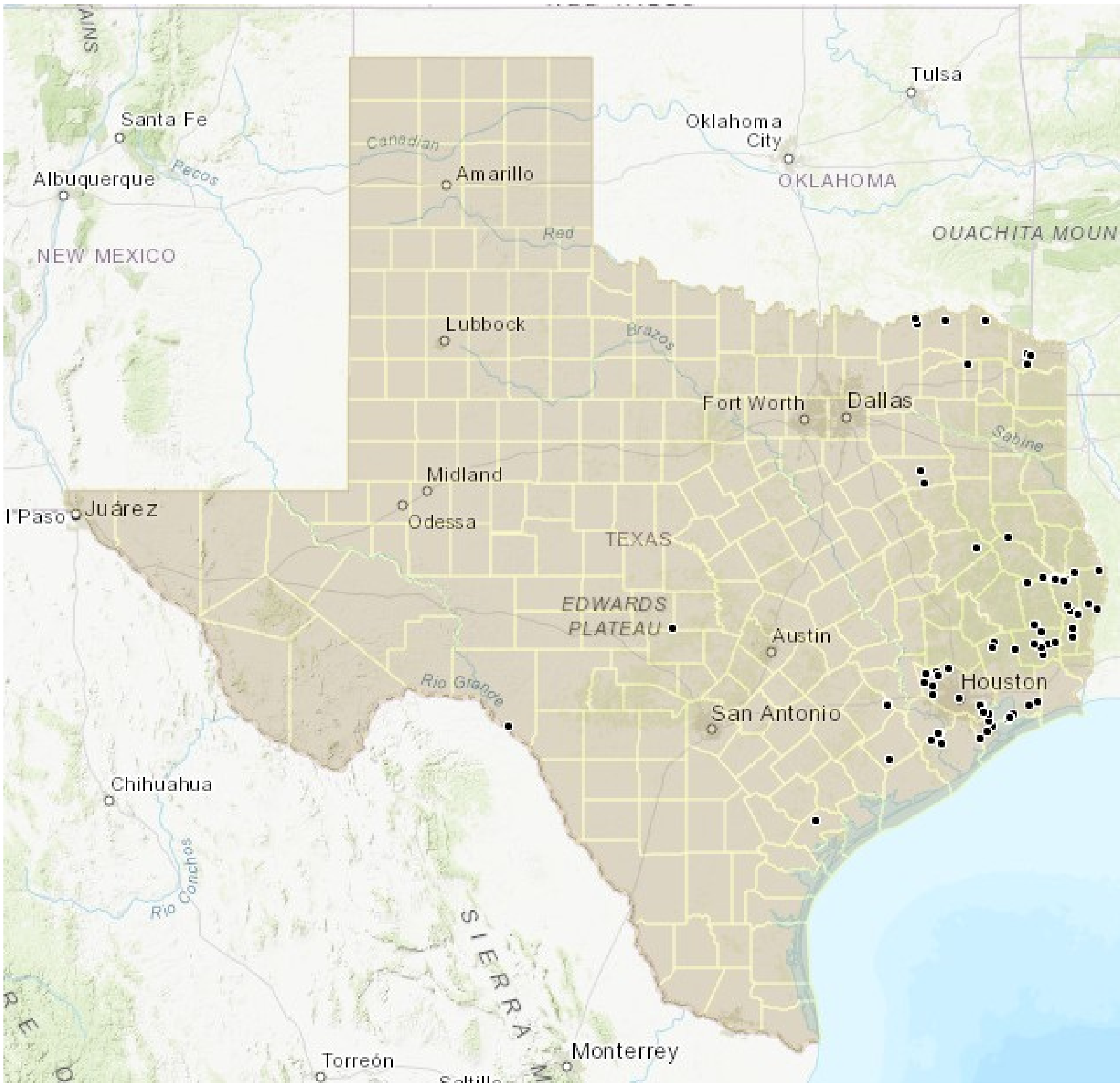




Asclepias longifolia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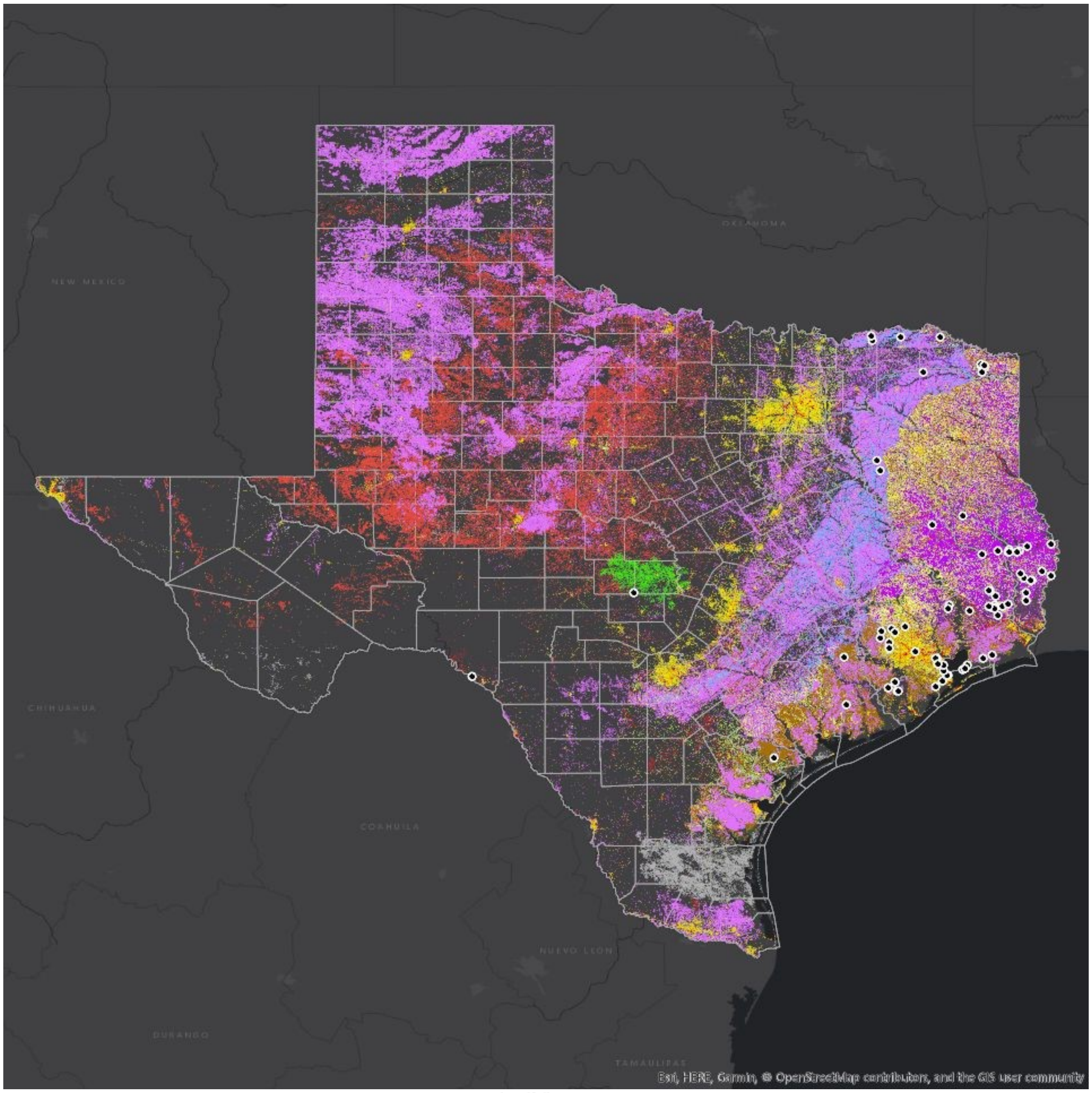
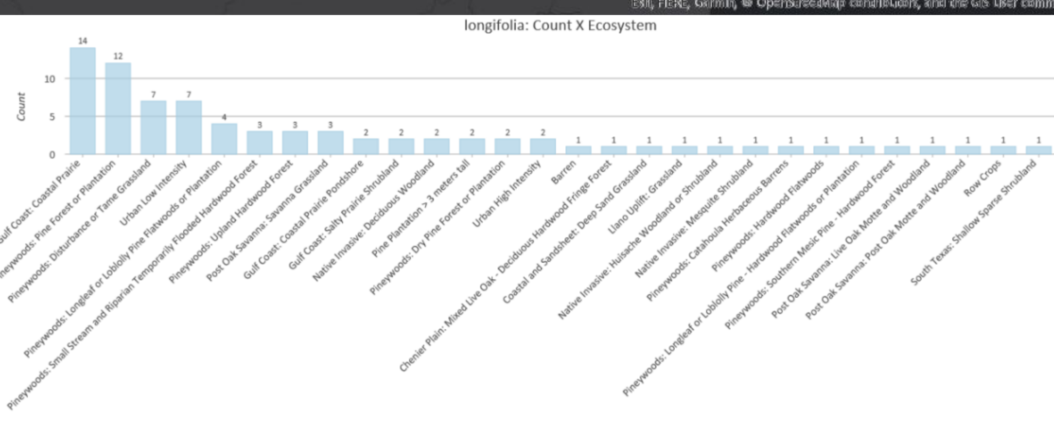
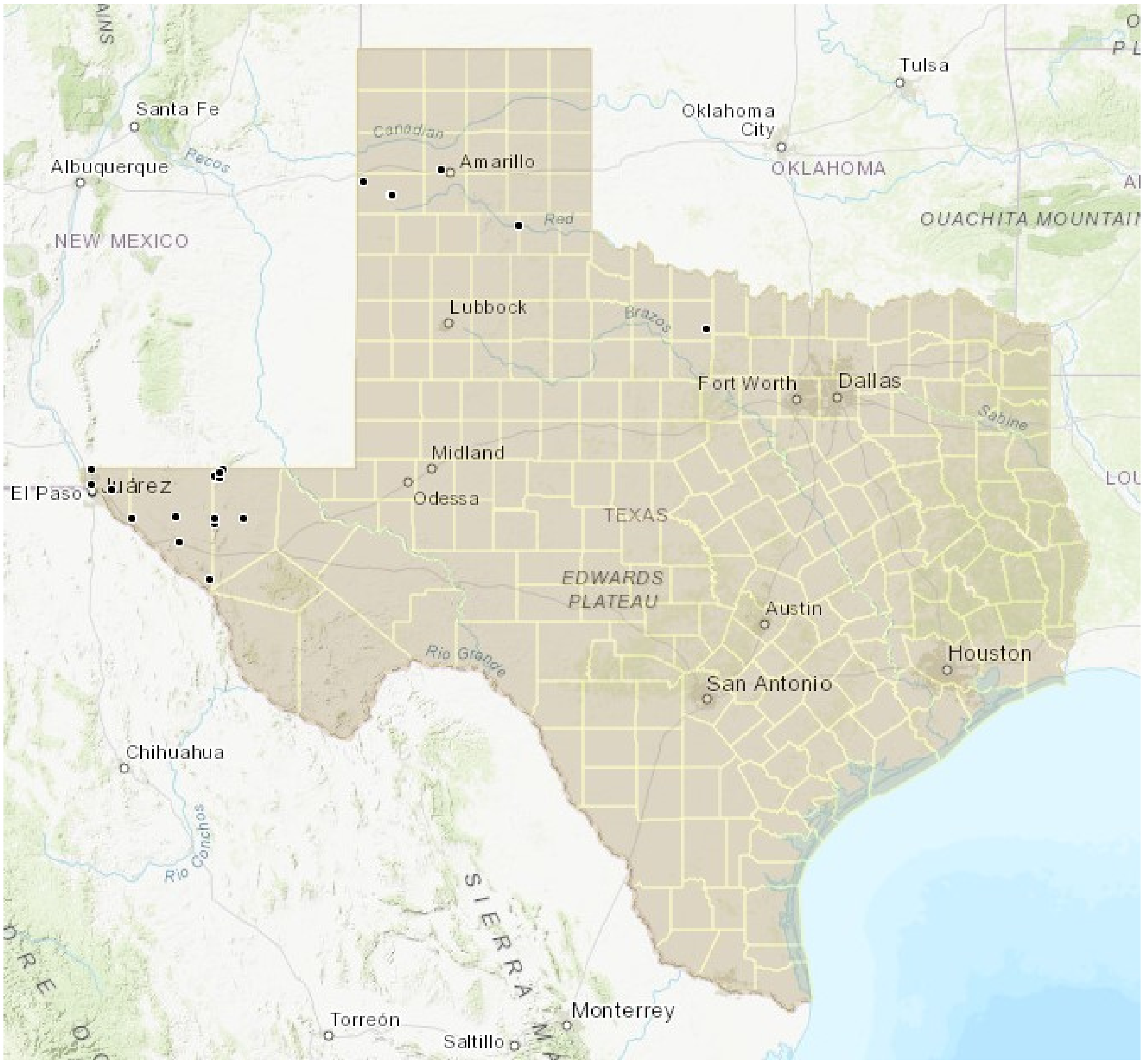




Asclepias macrotis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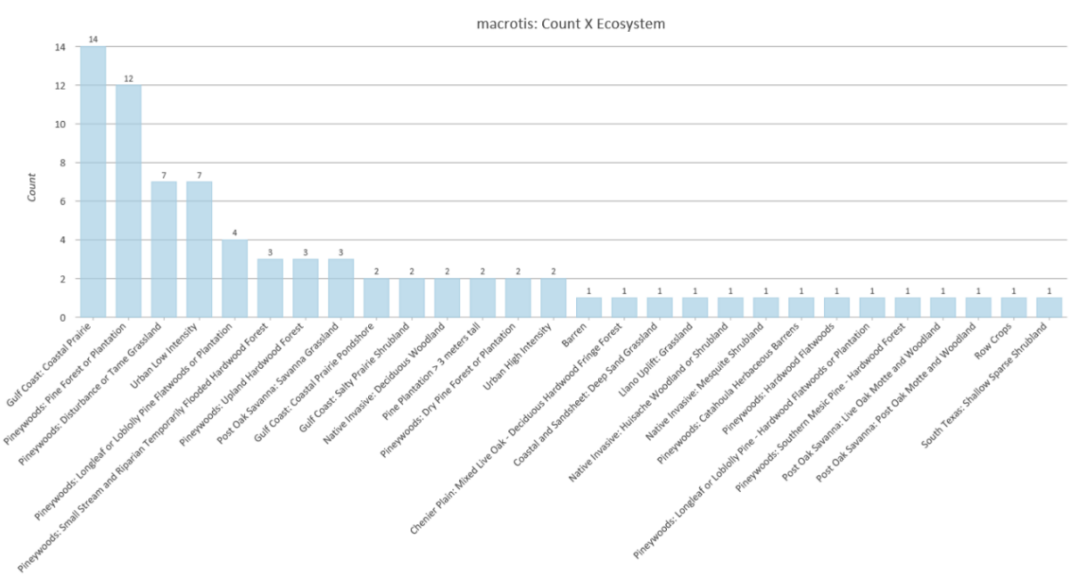
macrotis: Count X Ecosystem
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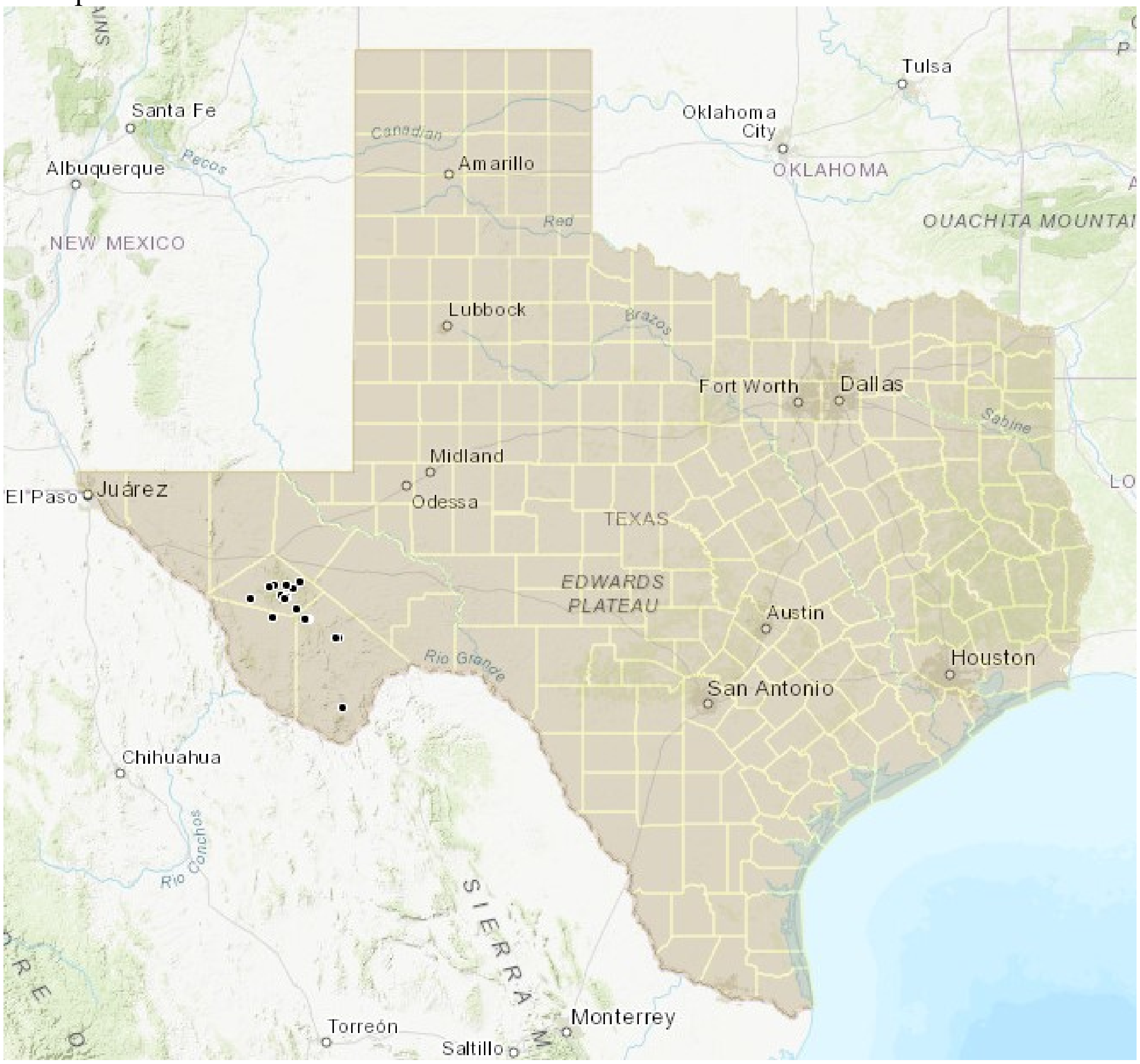
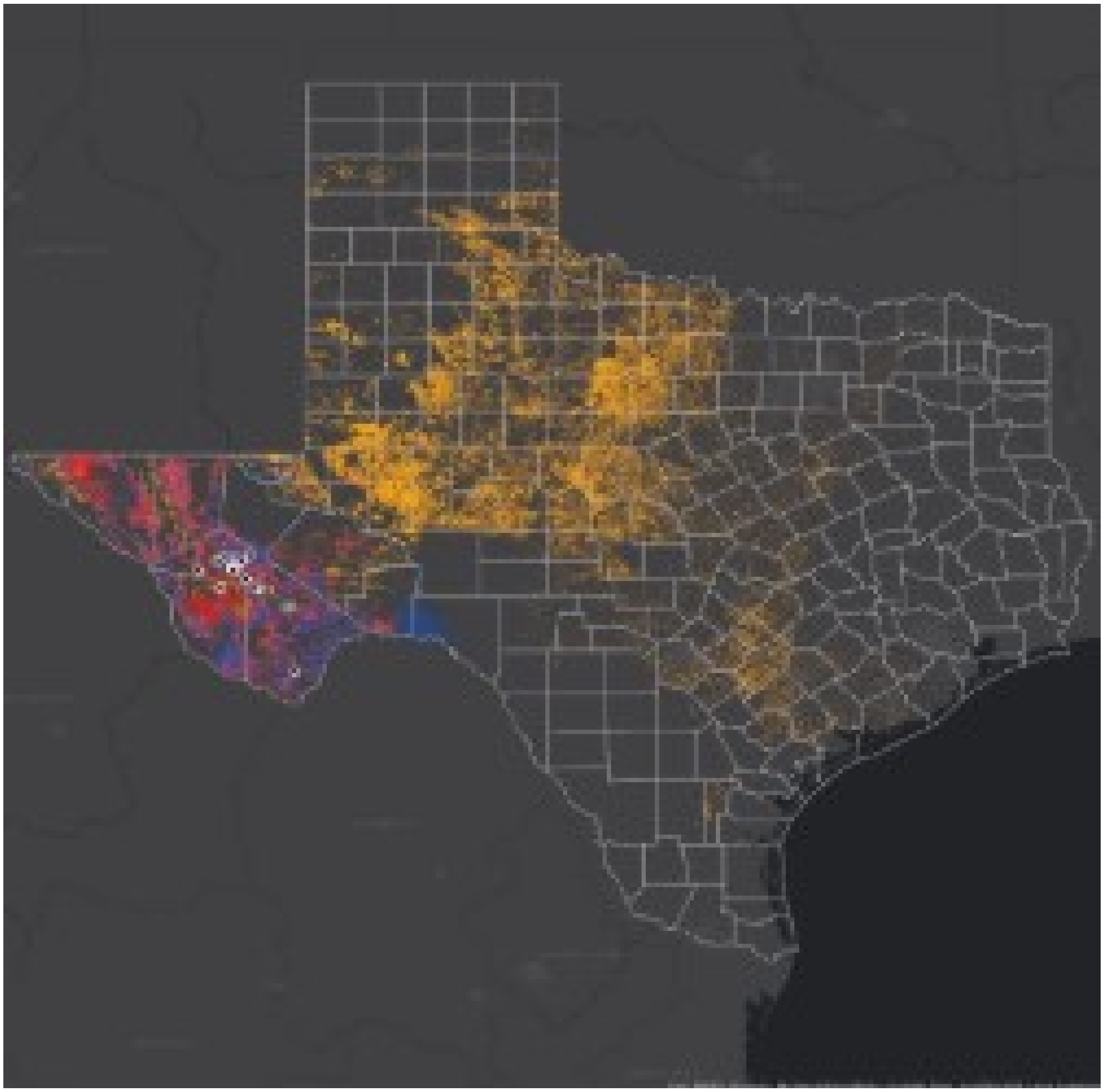
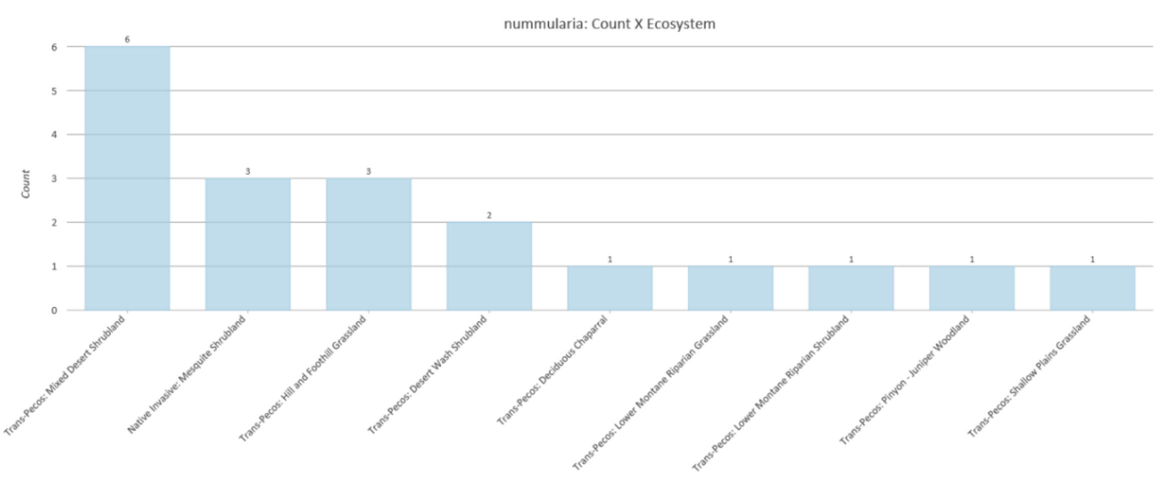
Gulf Coast: Coastal Prairie
Pineywoods: Pine Forest or Plantation
Pineywoods: Disturbance or Tame Grassland
Urban Low Intensity
Pineywoods: Longleaf or Loblolly Pine Flatwoods or Plantation
Pineywoods: Small Stream and Riparian Temporarily Flooded Hardwood Forest
Pineywoods: Upland Hardwood Forest
Post Oak Savanna: Savanna Grassland
Gulf Coast: Coastal Prairie Pondshore
Gulf Coast: Salty Prairie Shrubland
Native Invasive: Deciduous Woodland
Pine Plantation > 3 meters tall
Pineywoods: Dry Pine Forest or Plantation
Urban High Intensity
Barren
Chenier Plain: Mixed Live Oak - Deciduous Hardwood Fringe Forest
Coastal and Sandsheet: Deep Sand Grassland
Llano Uplift: Grassland
Native Invasive: Huisache Woodland or Shrubland
Native Invasive: Mesquite Shrubland
Pineywoods: Catahoula Herbaceous Barrens
Pineywoods: Hardwood Flatwoods
Pineywoods: Longleaf or Loblolly Pine - Hardwood Flatwoods or Plantation
Pineywoods: Southern Mesic Pine - Hardwood Forest
Post Oak Savanna: Live Oak Motte and Woodland
Post Oak Savanna: Post Oak Motte and Woodland
Row Crops
South Texas: Shallow Sparse Shrubland
Common Name

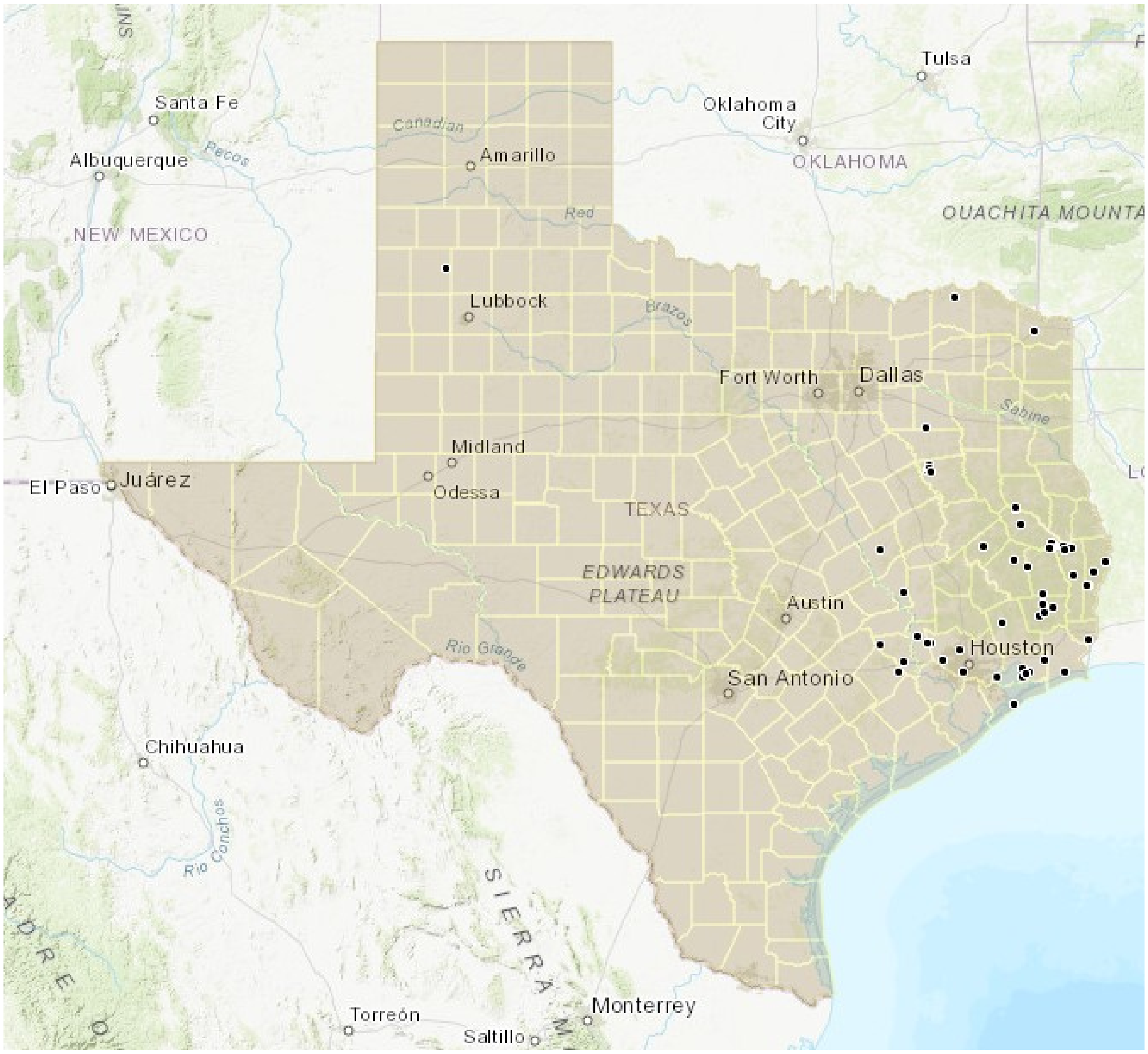
Asclepias nummularia







Asclepias obovata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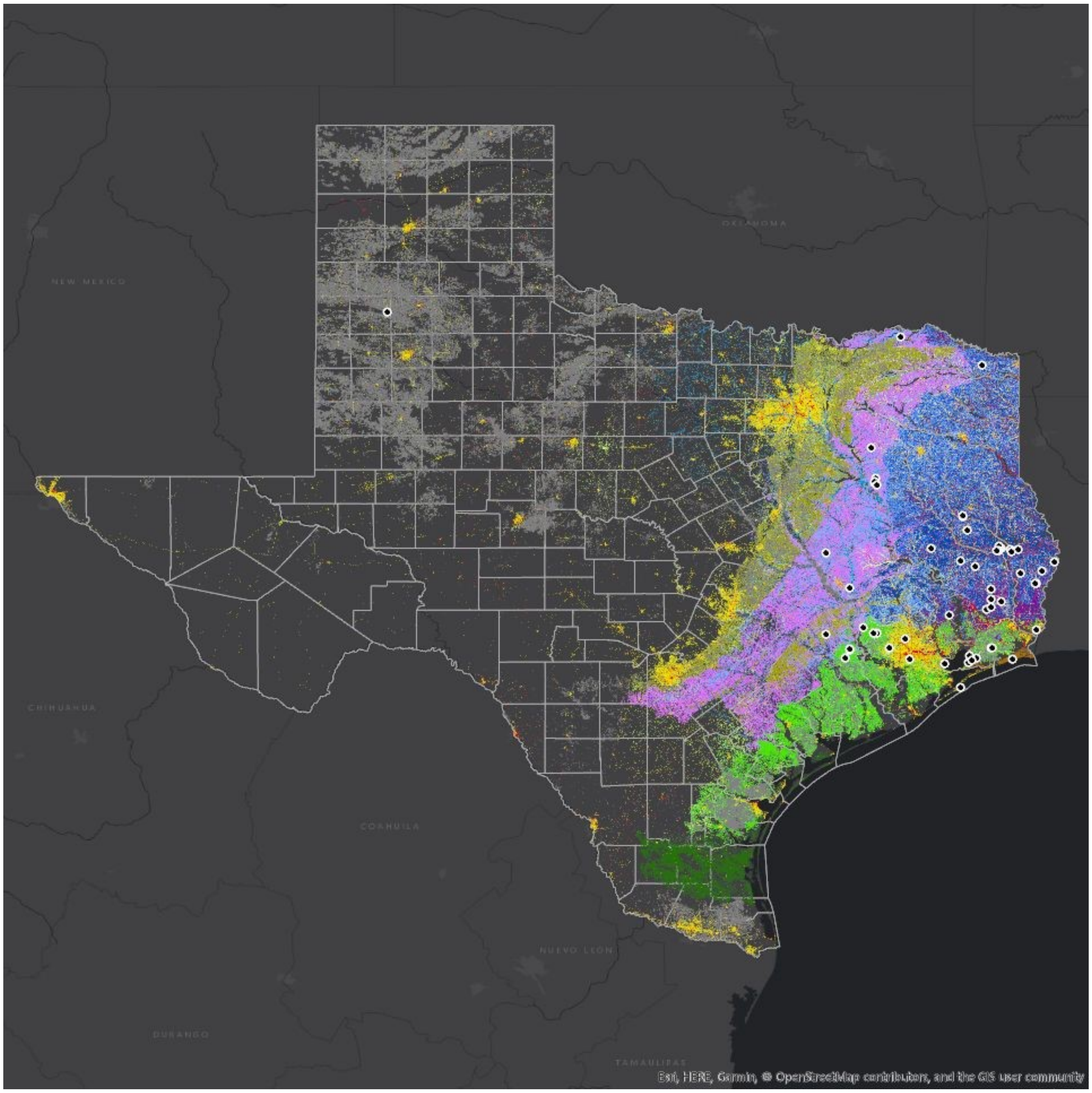
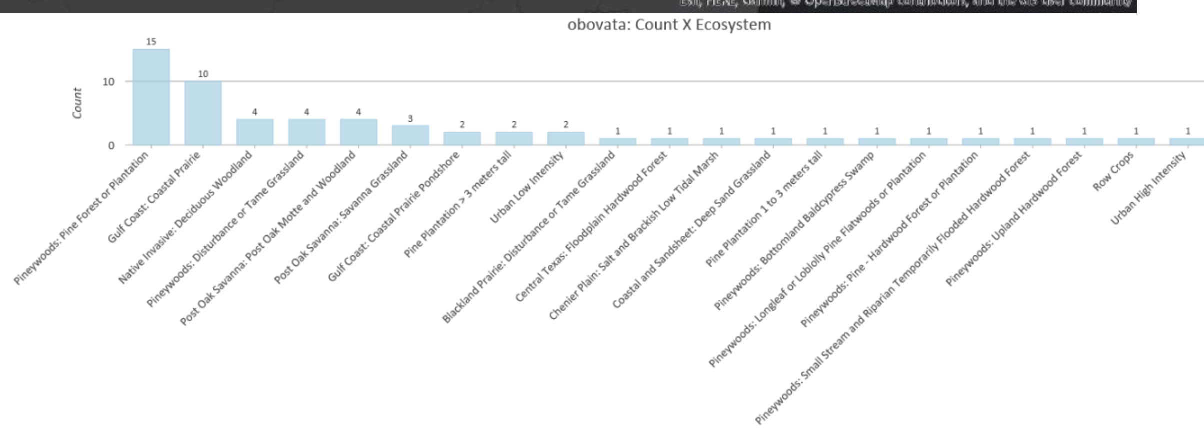
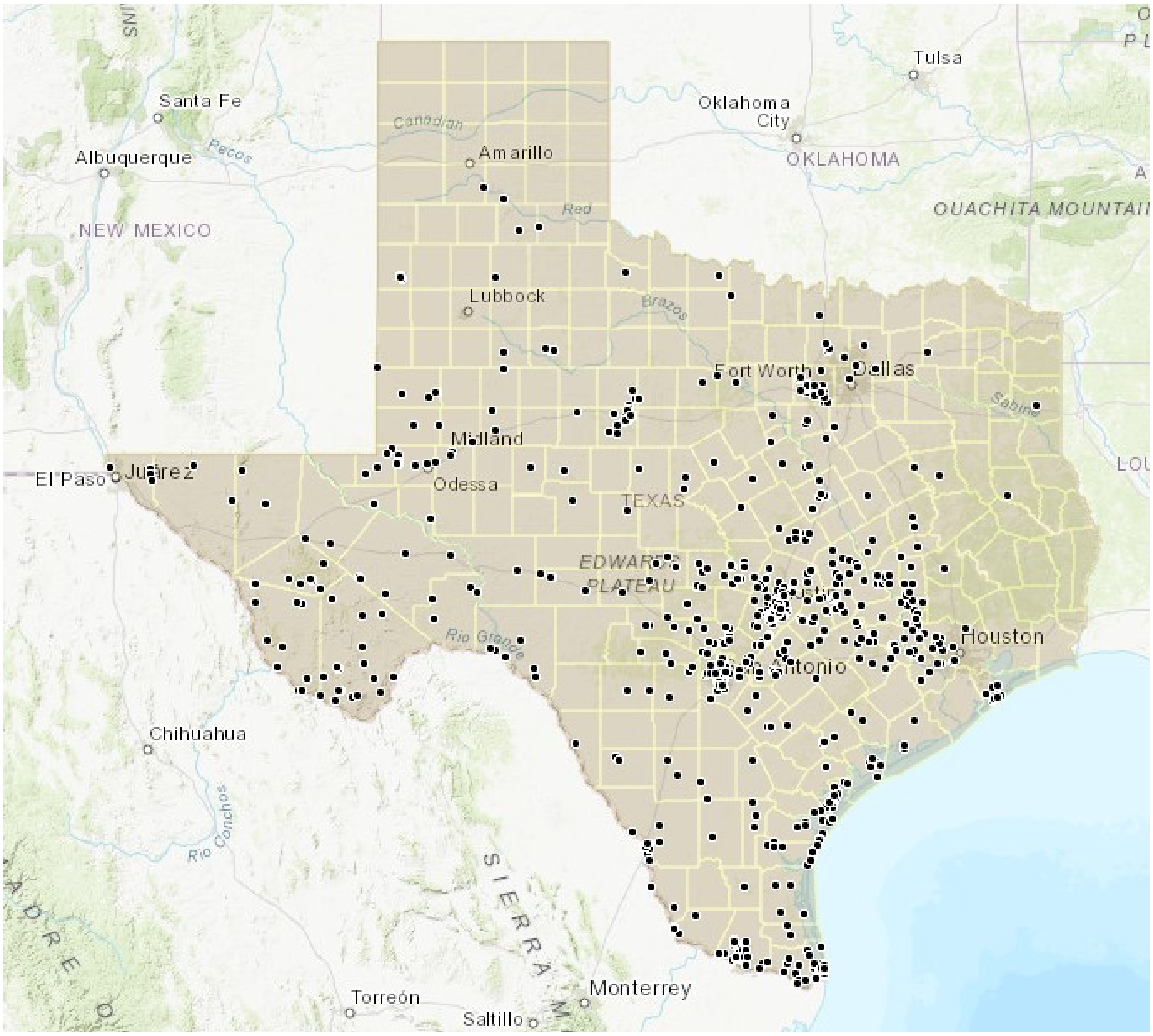




Asclepias oenotherioides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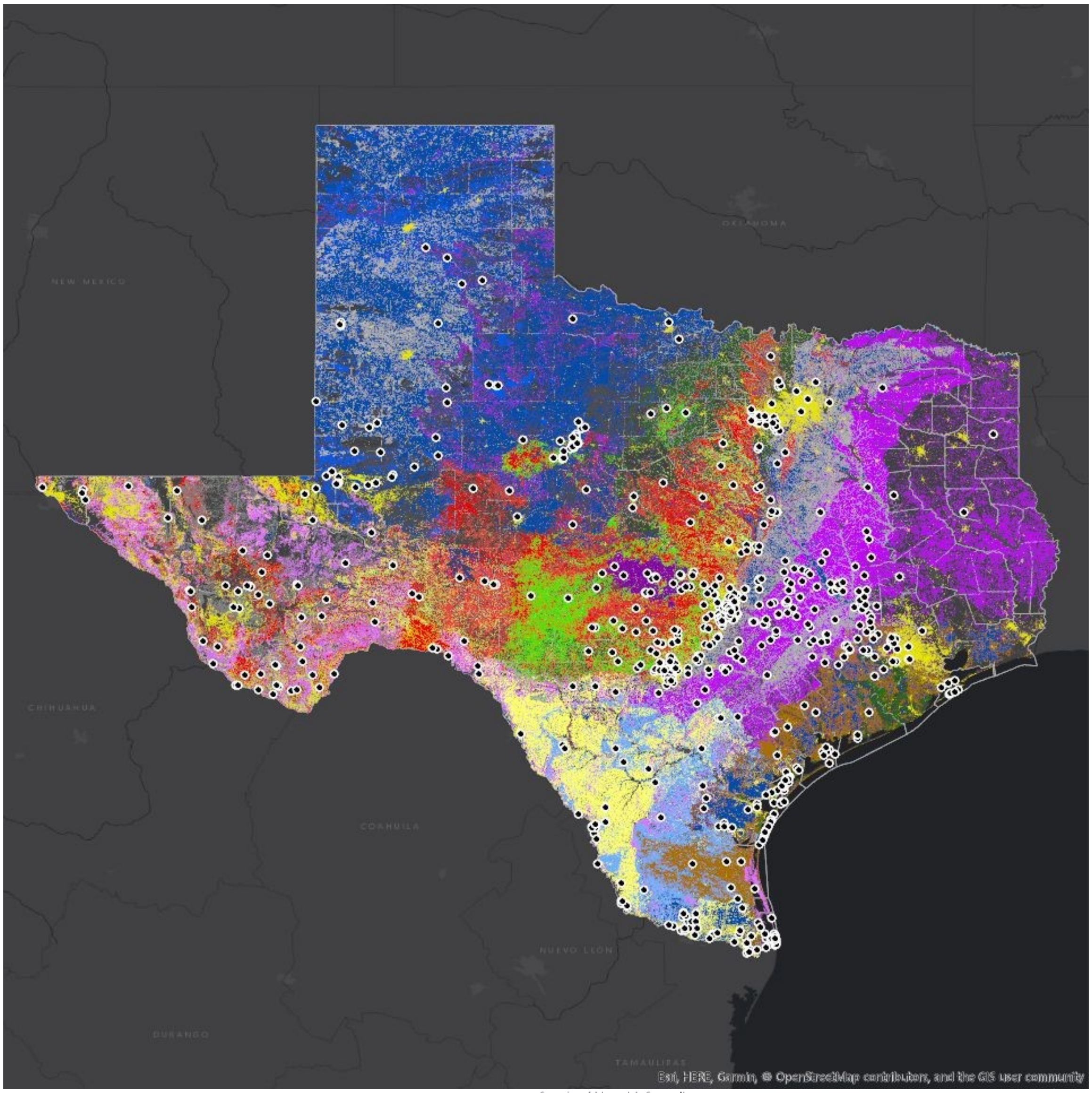
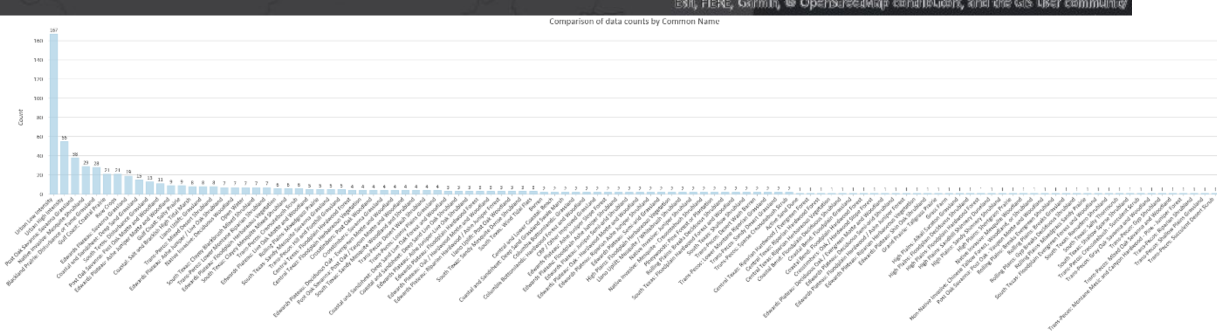
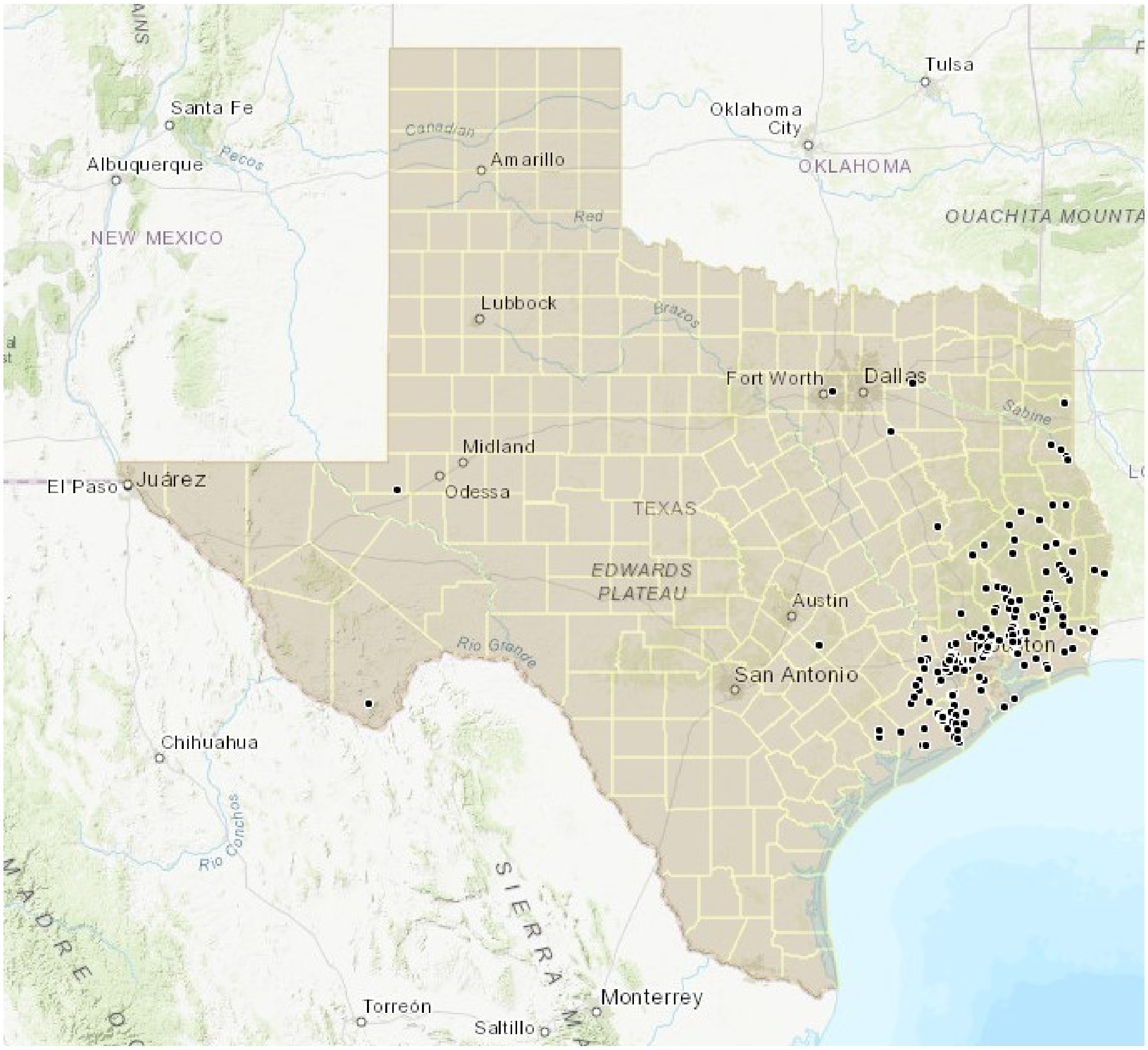




Asclepias perennis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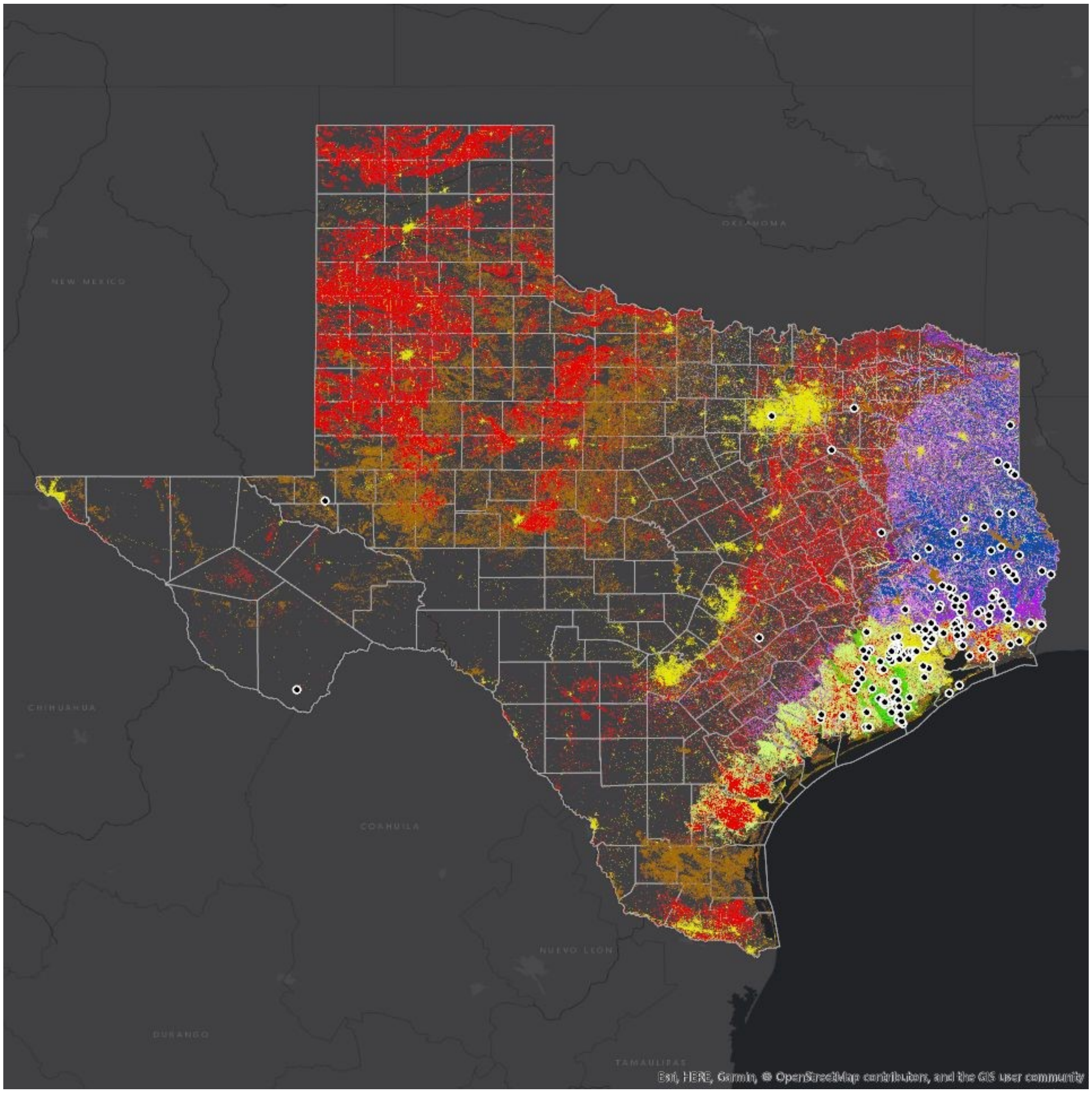
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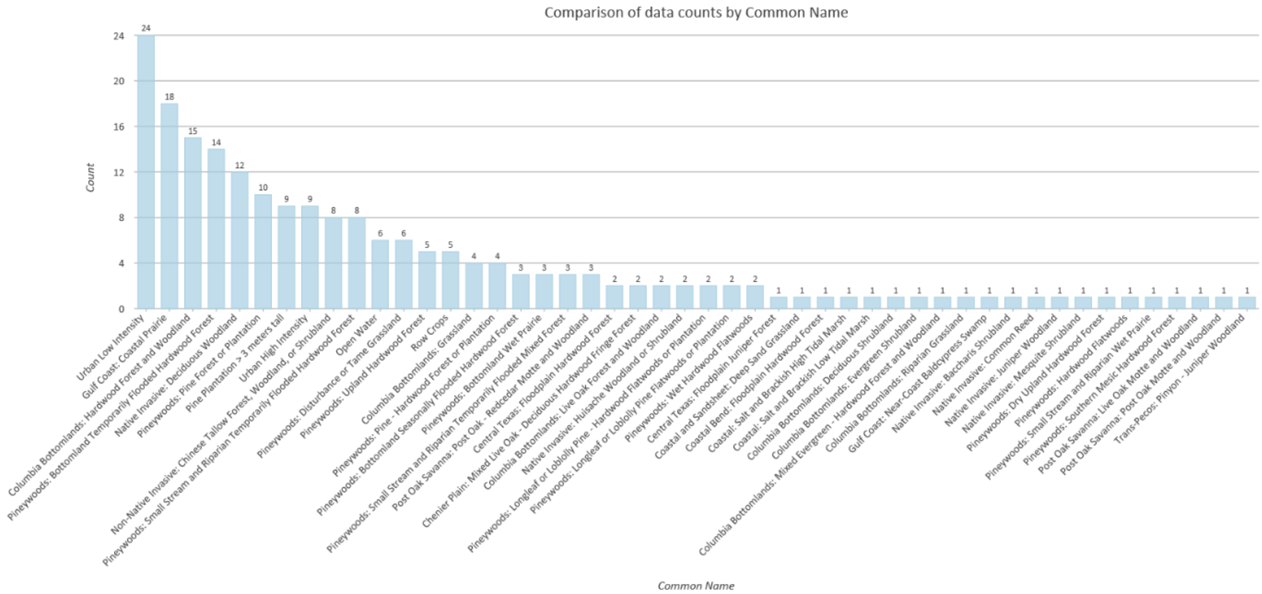

Asclepias prostrata

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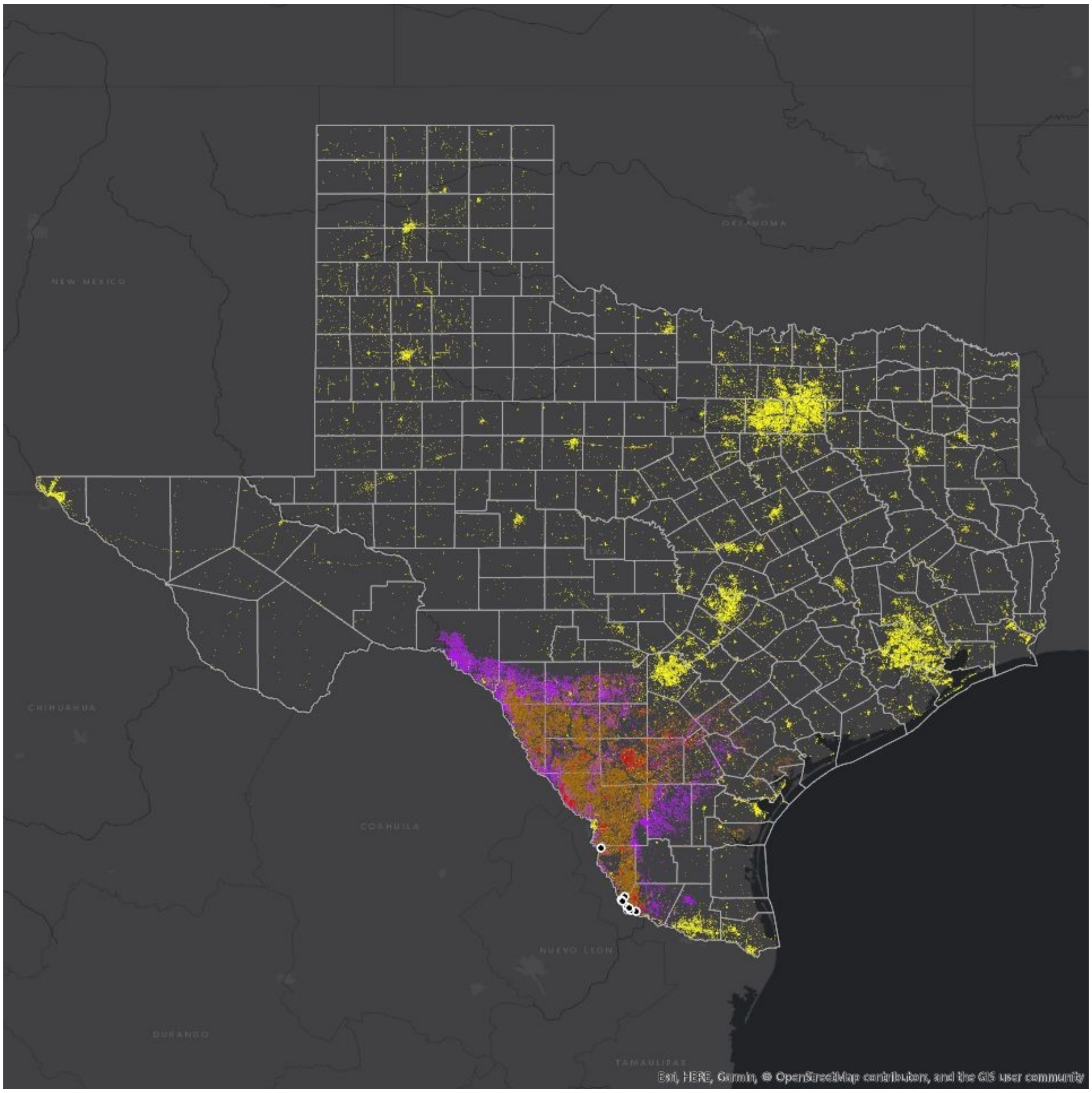
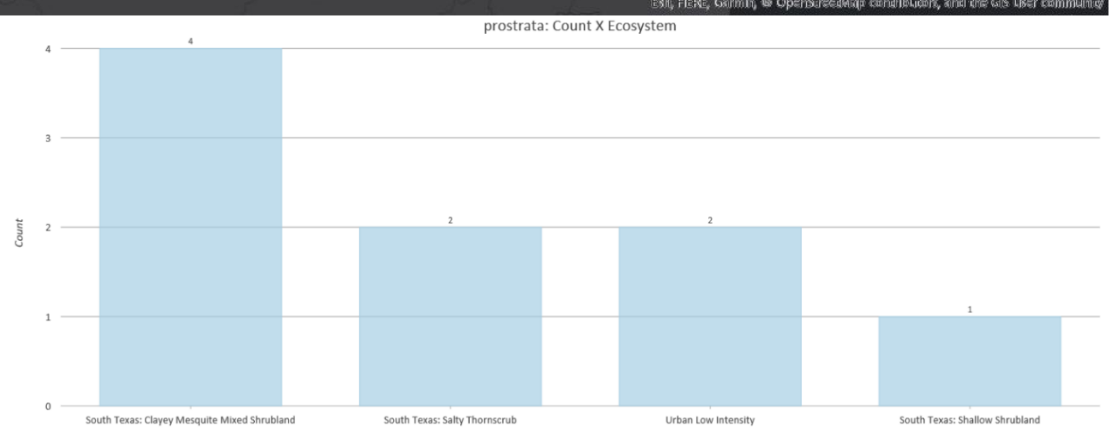
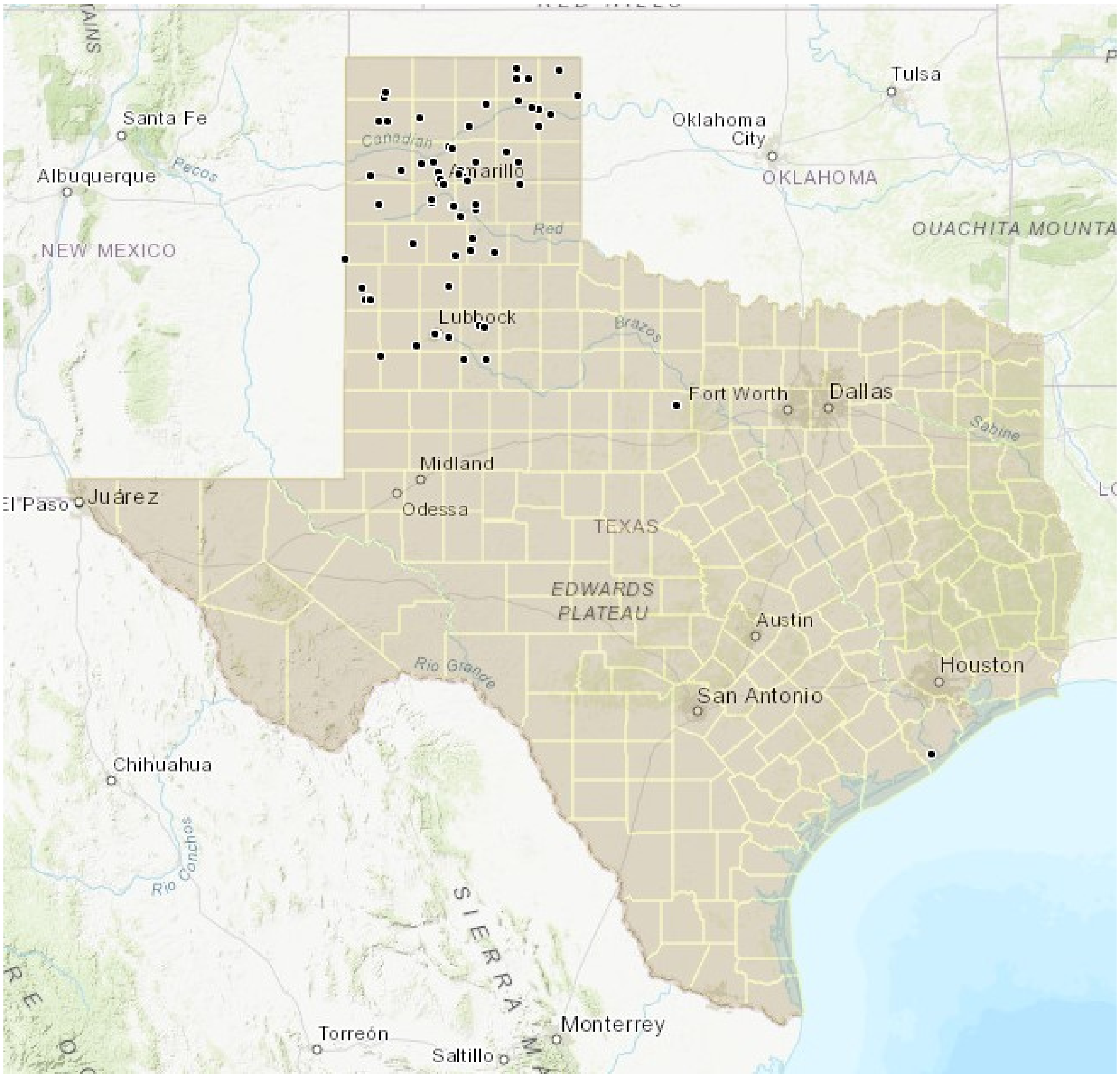




Asclepias pumila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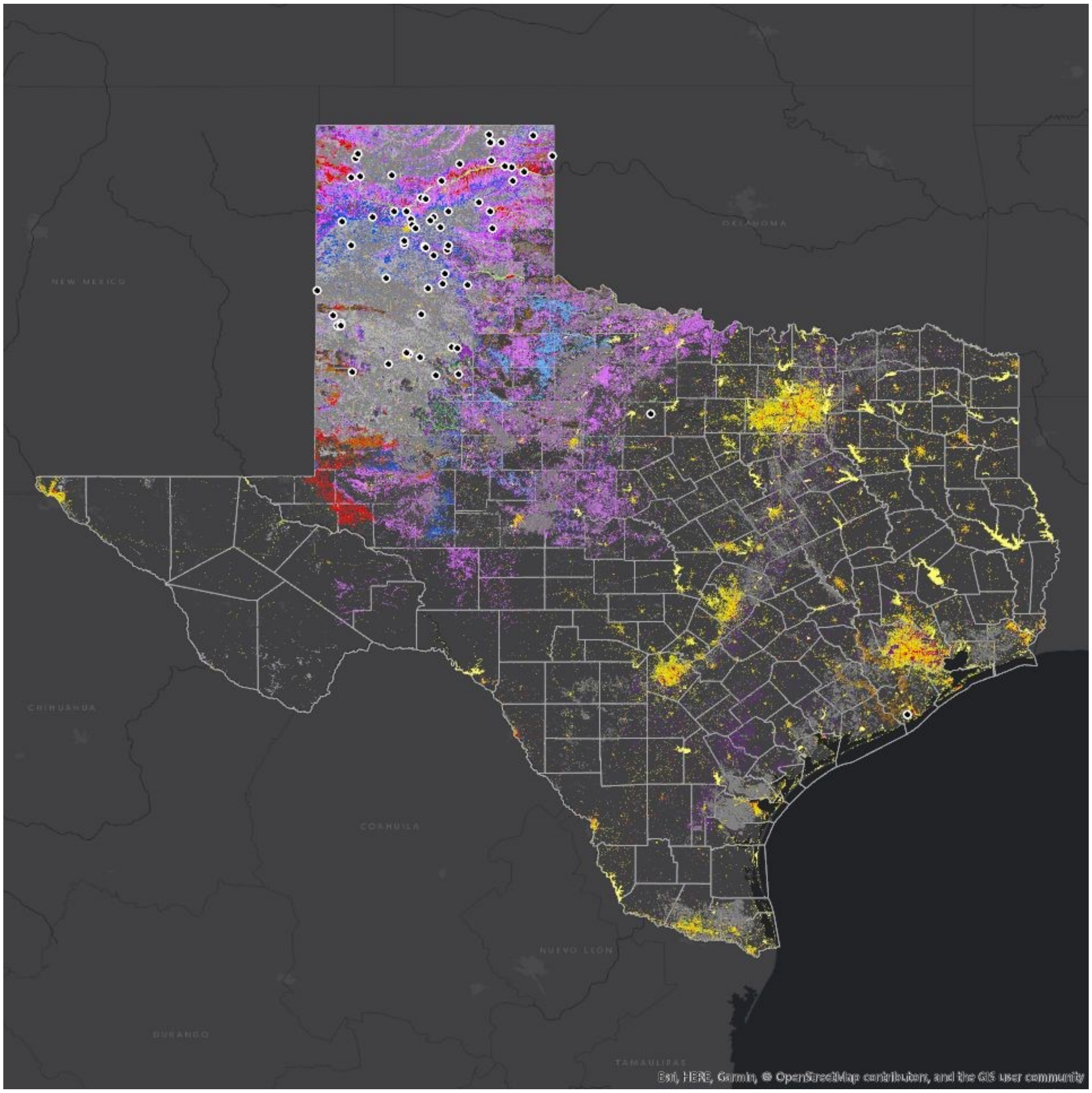

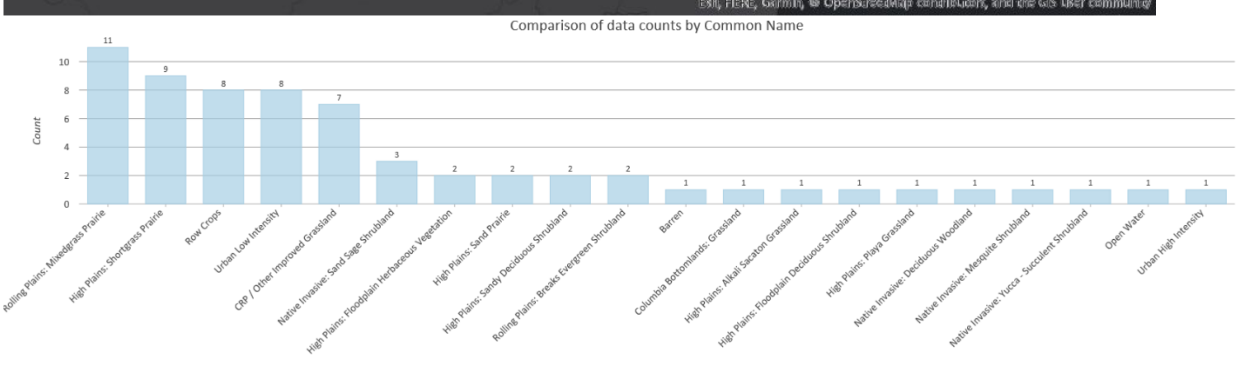

Asclepias purpurascens We're going to drop this species off the list. It appears to be extirpated .

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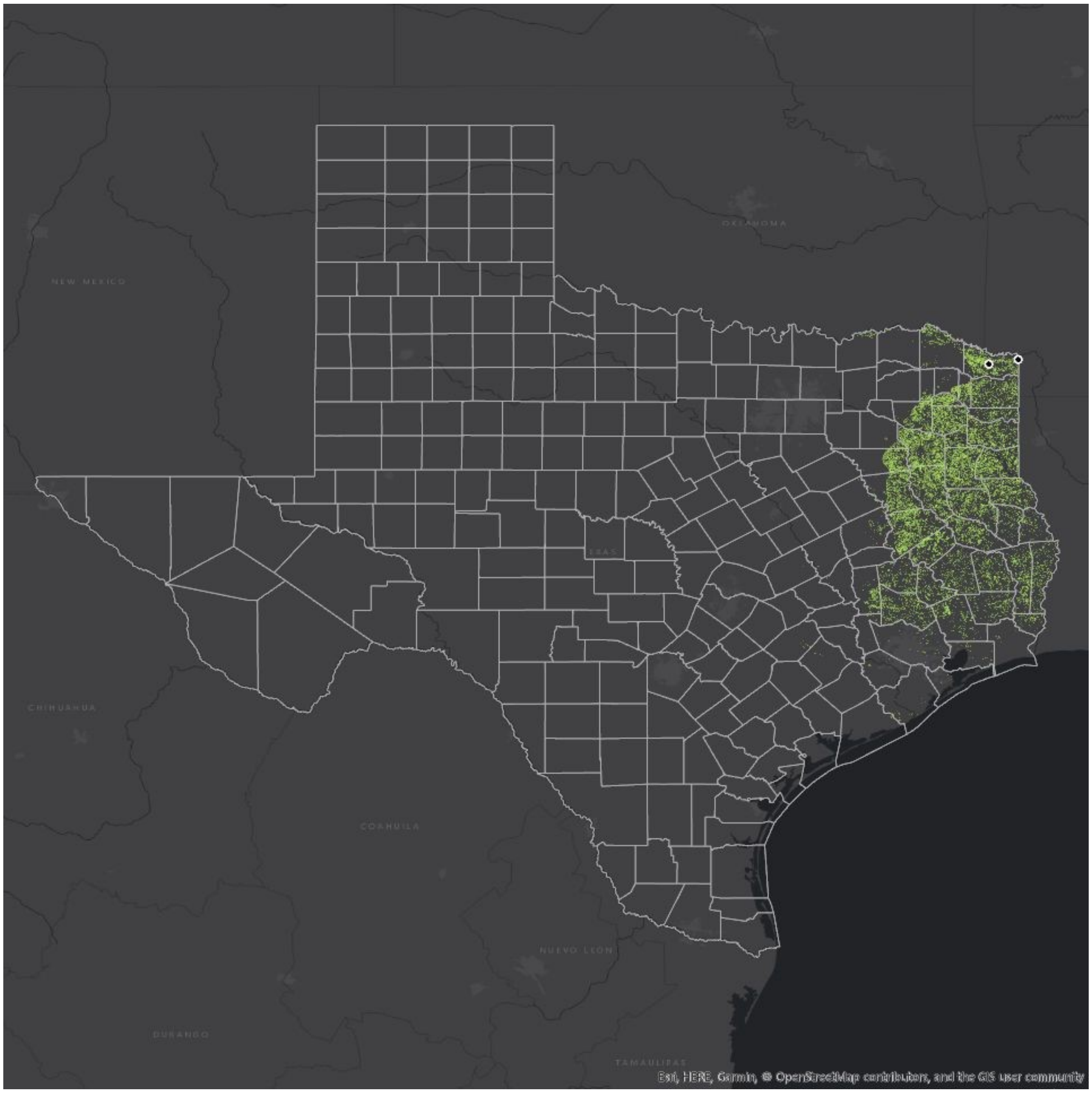
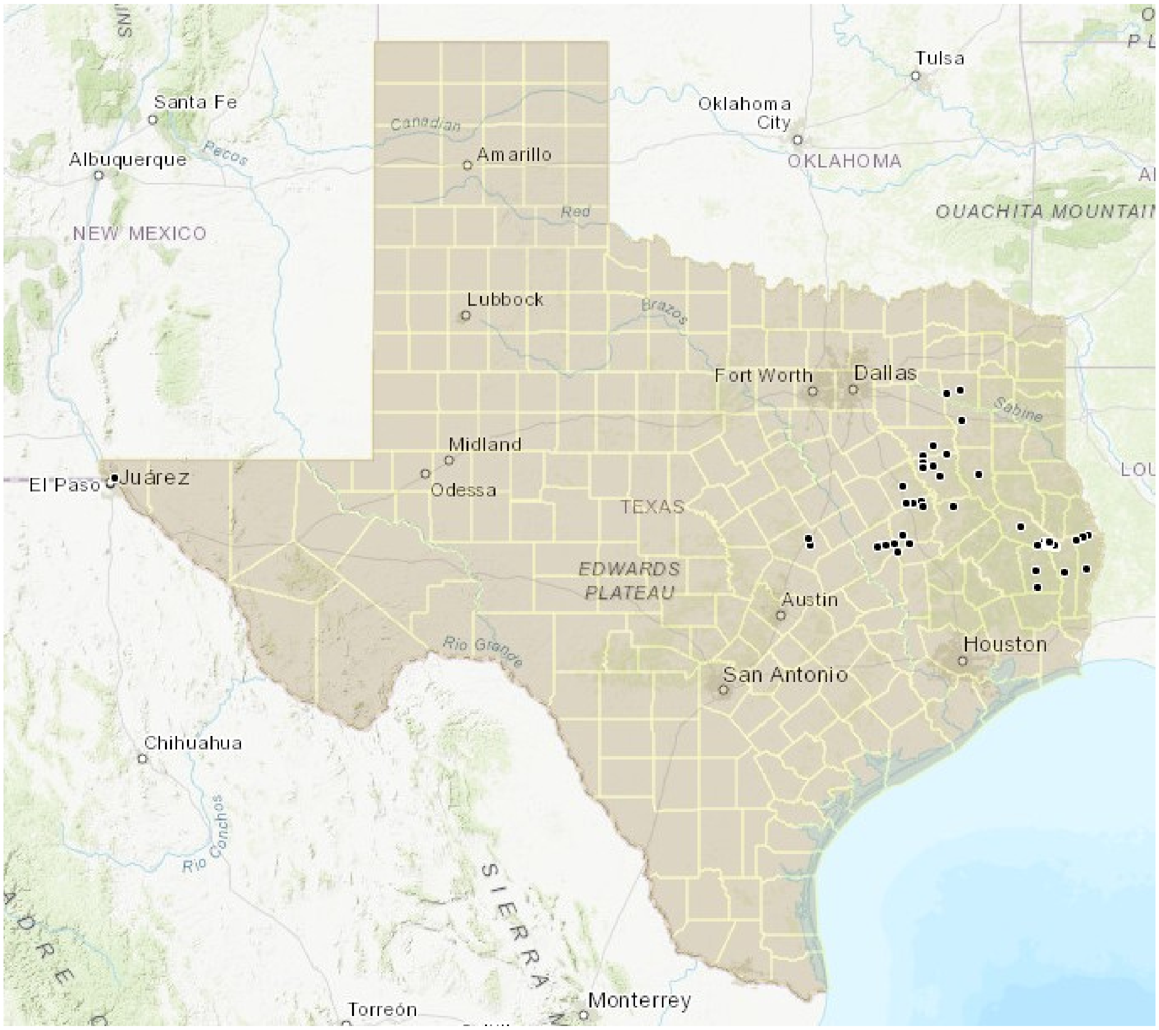


Asclepias rubra



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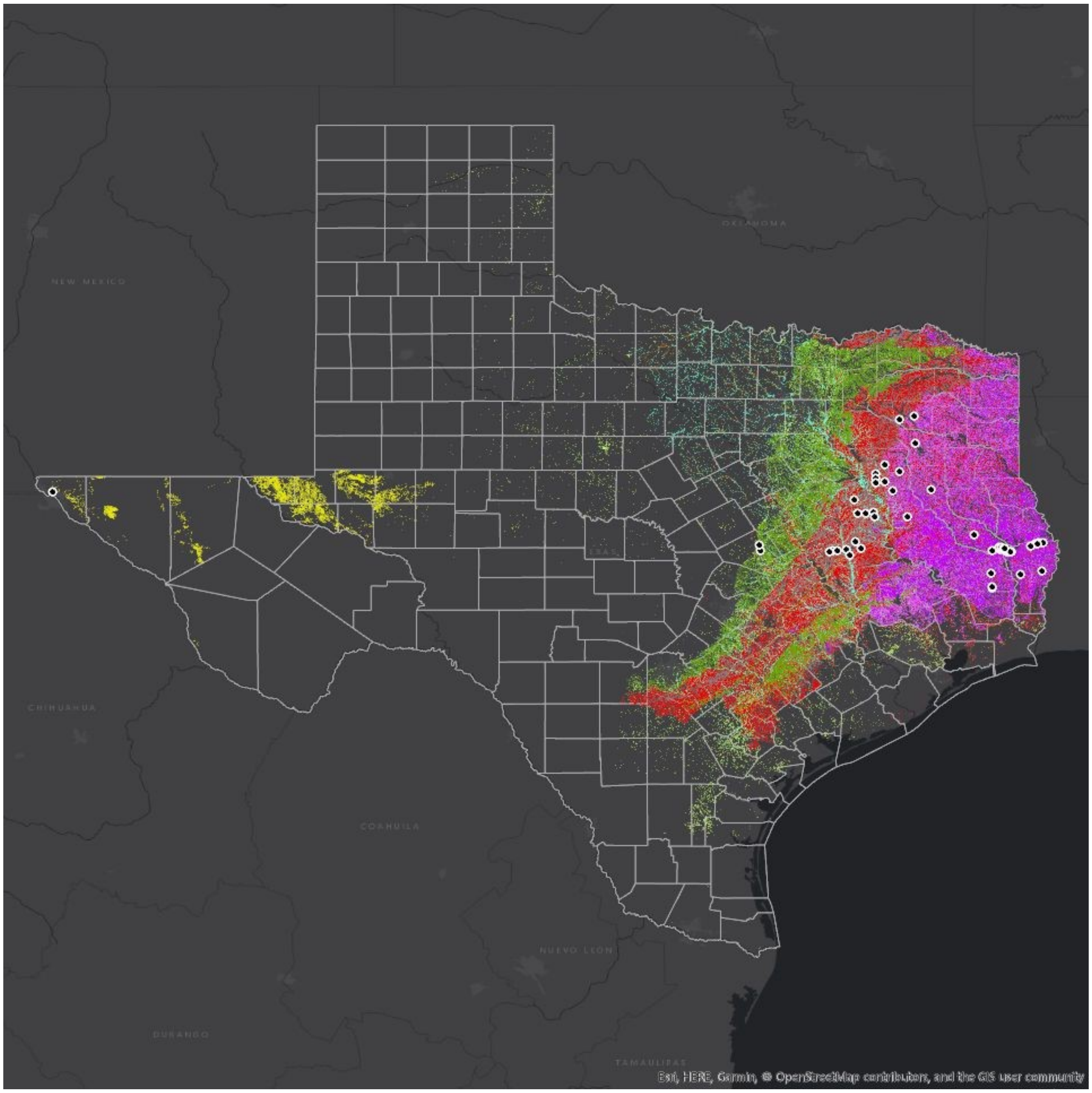
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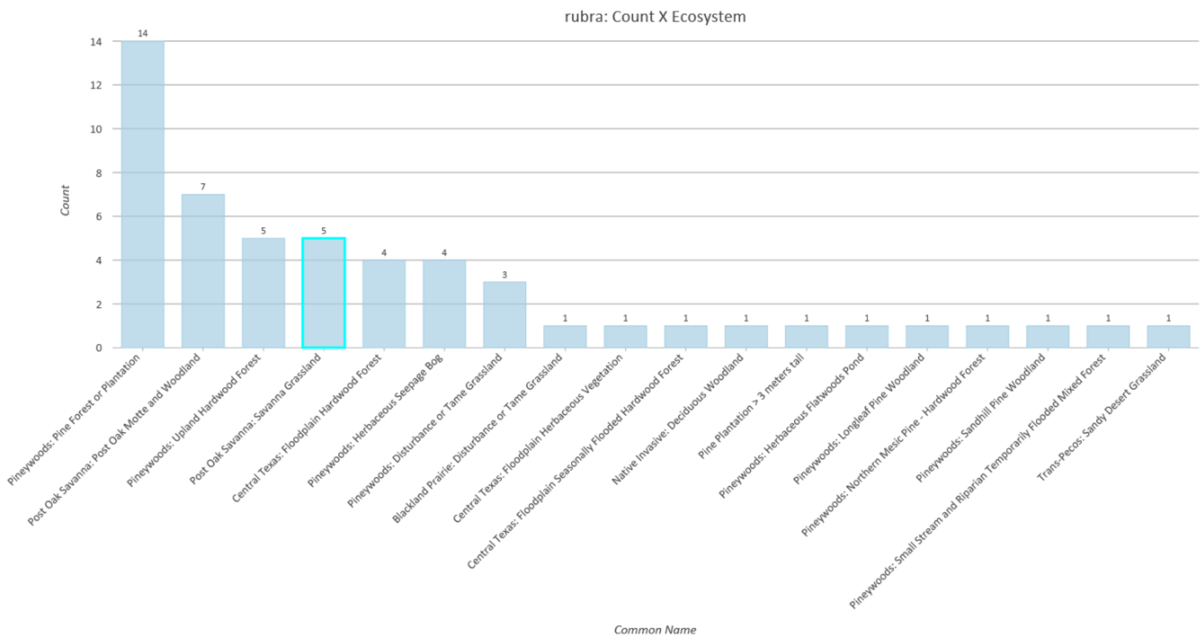
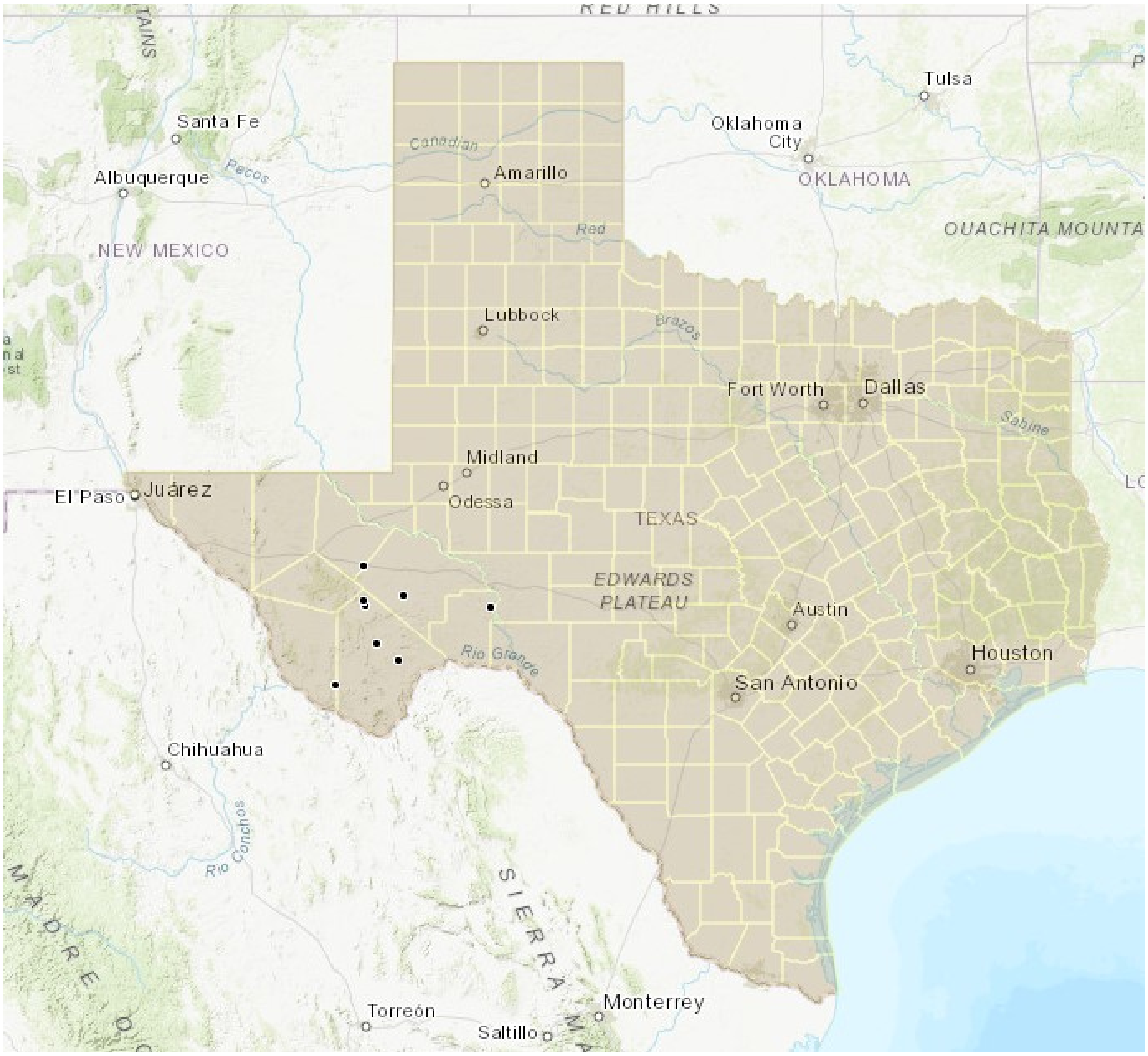


Asclepias scaposa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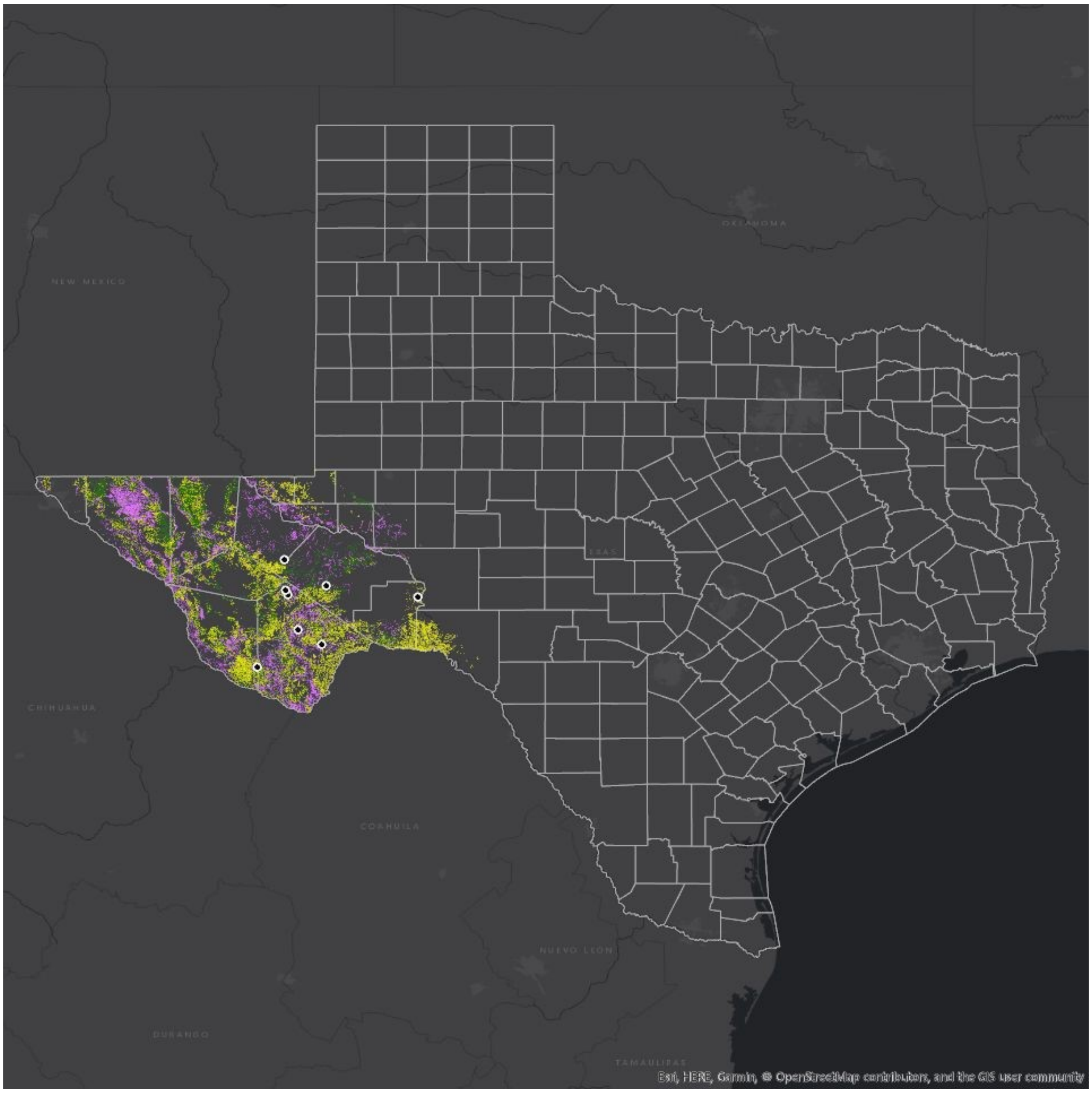
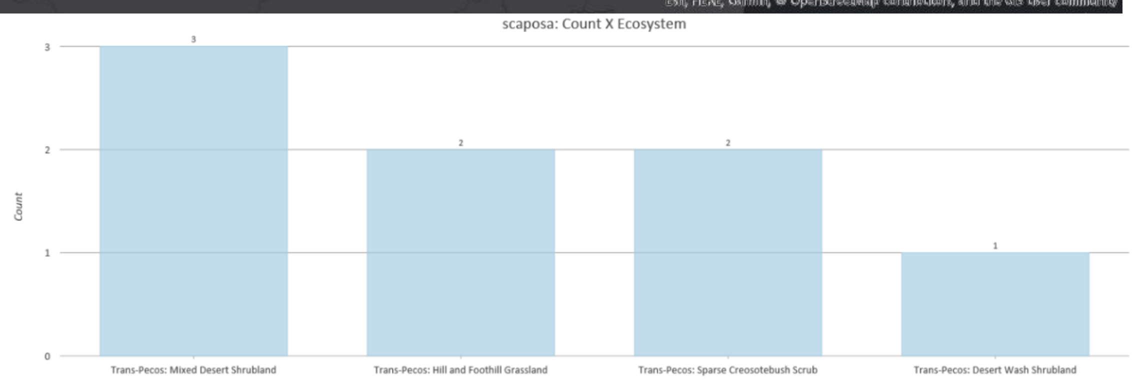
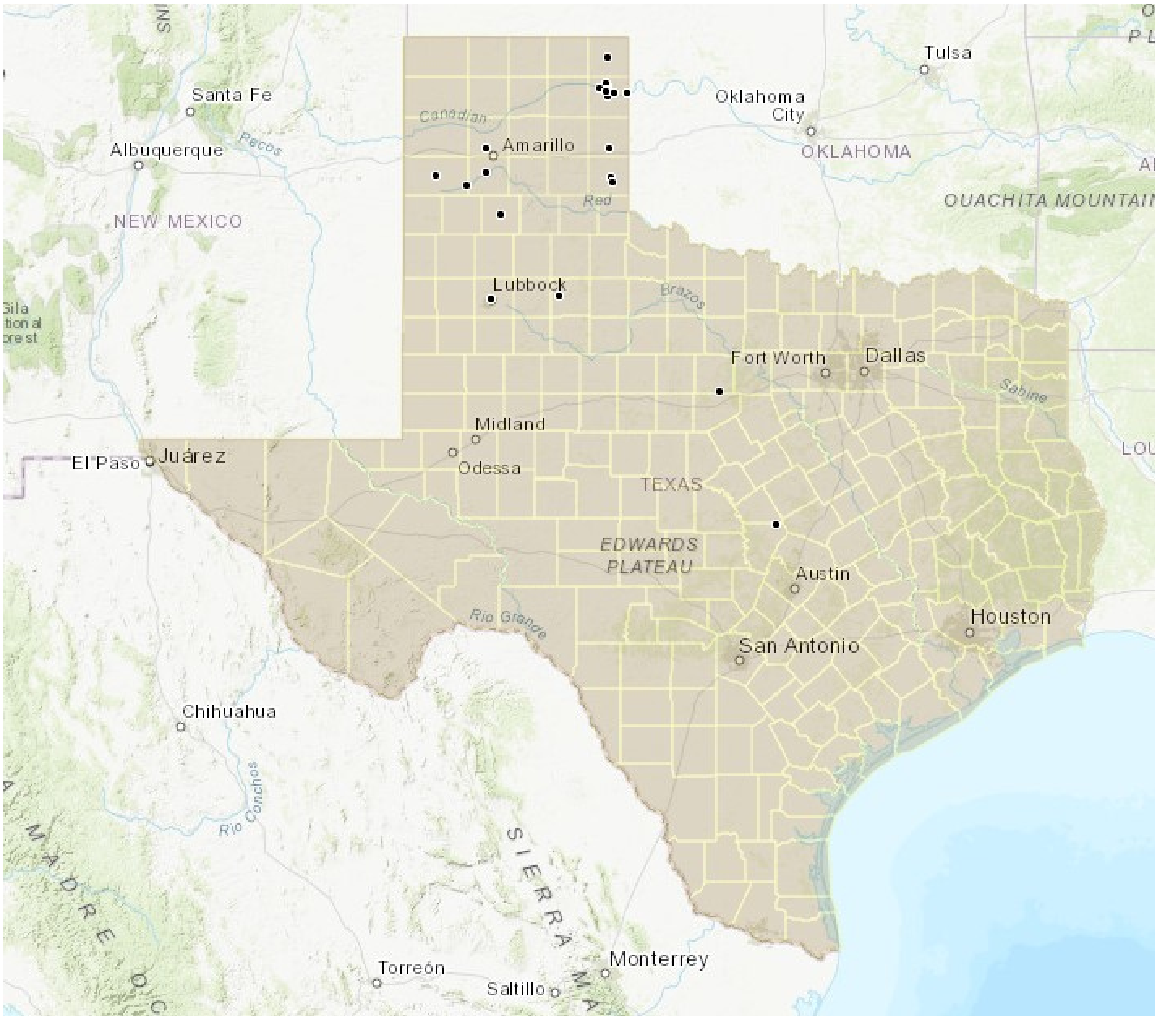




Asclepias speciosa



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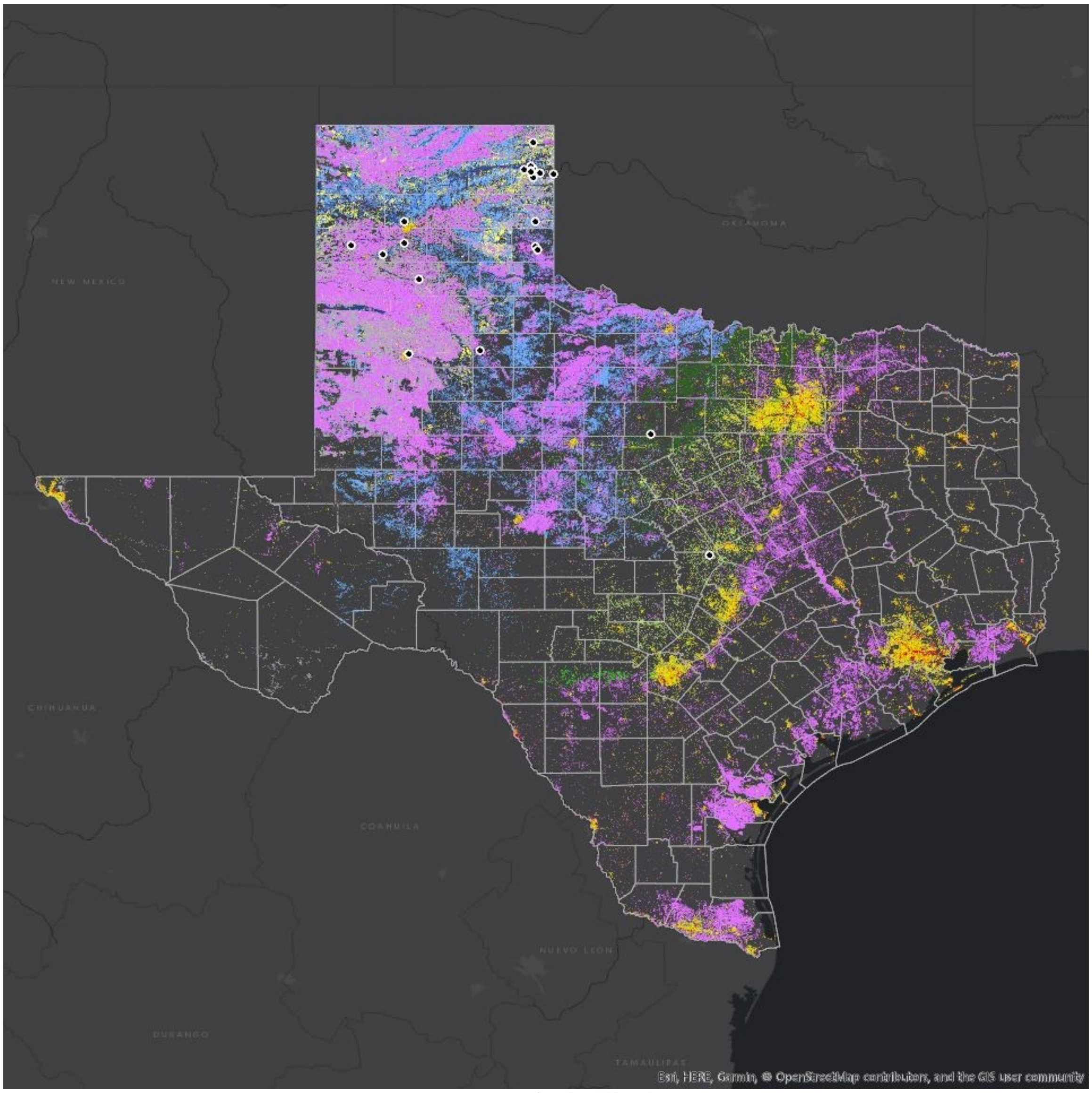
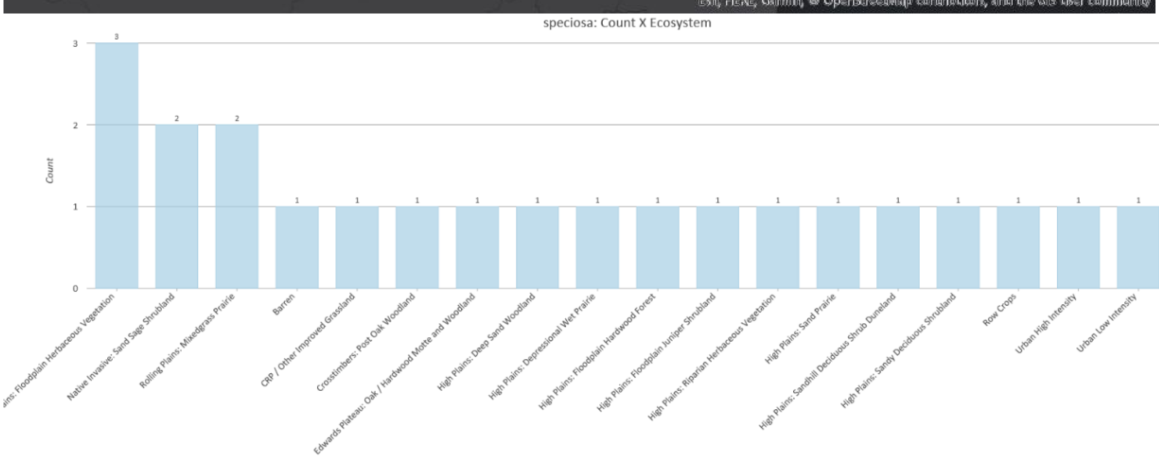
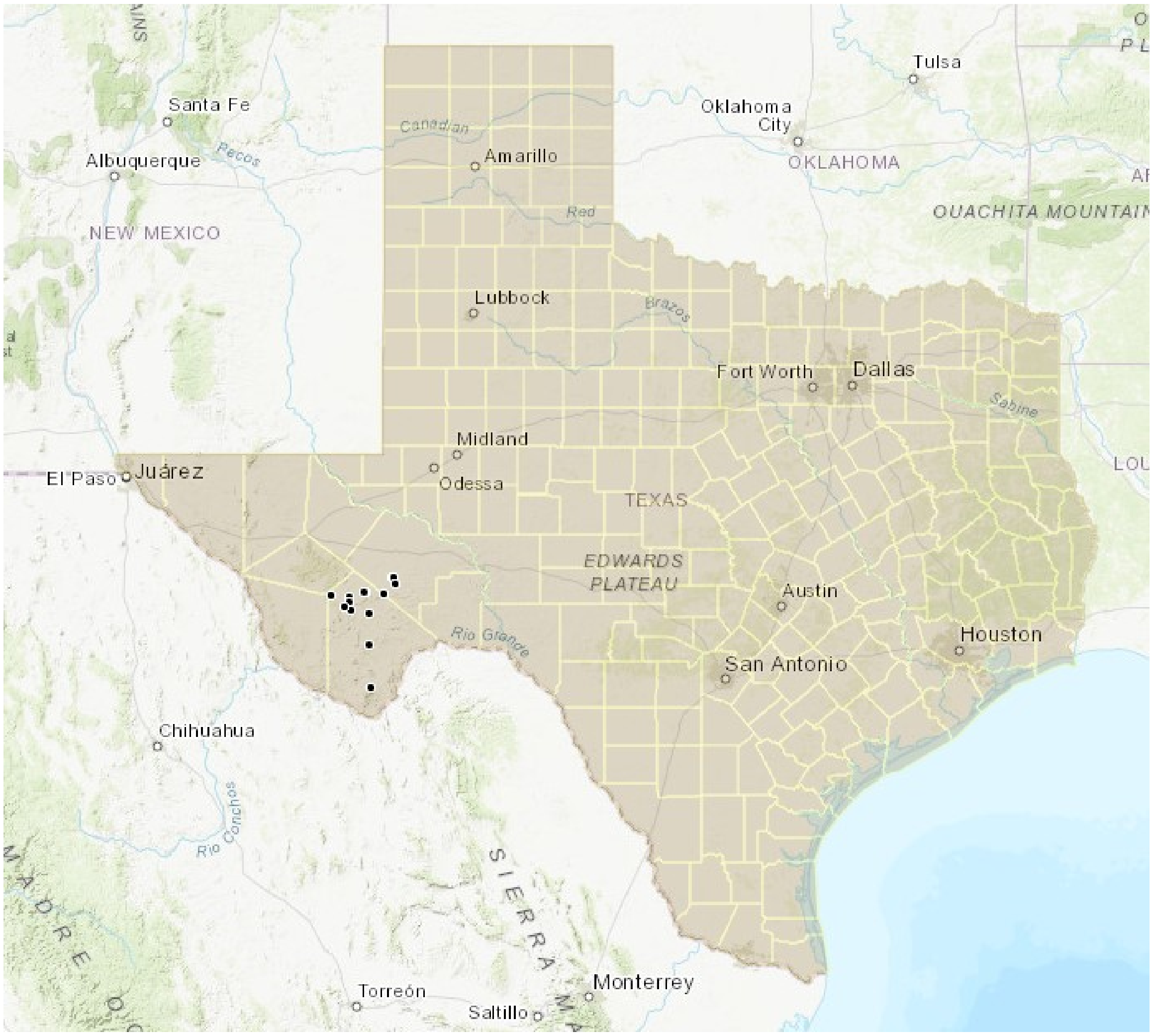




Asclepias sperryi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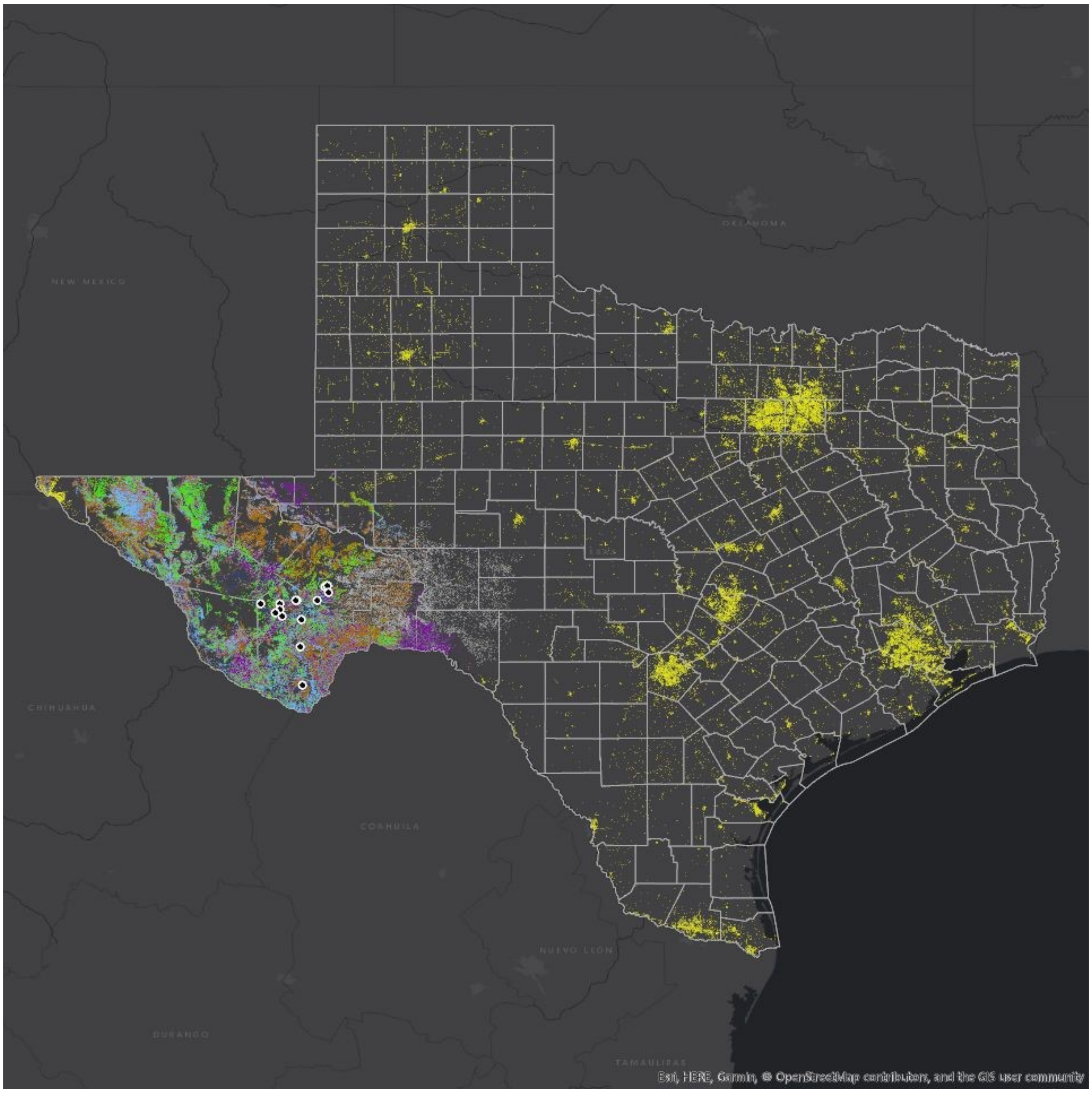

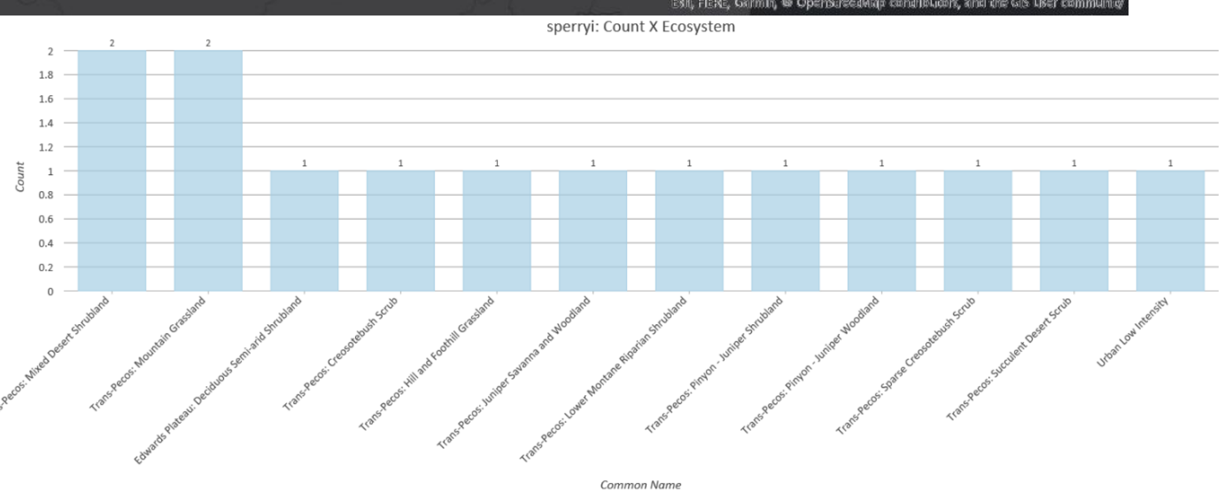

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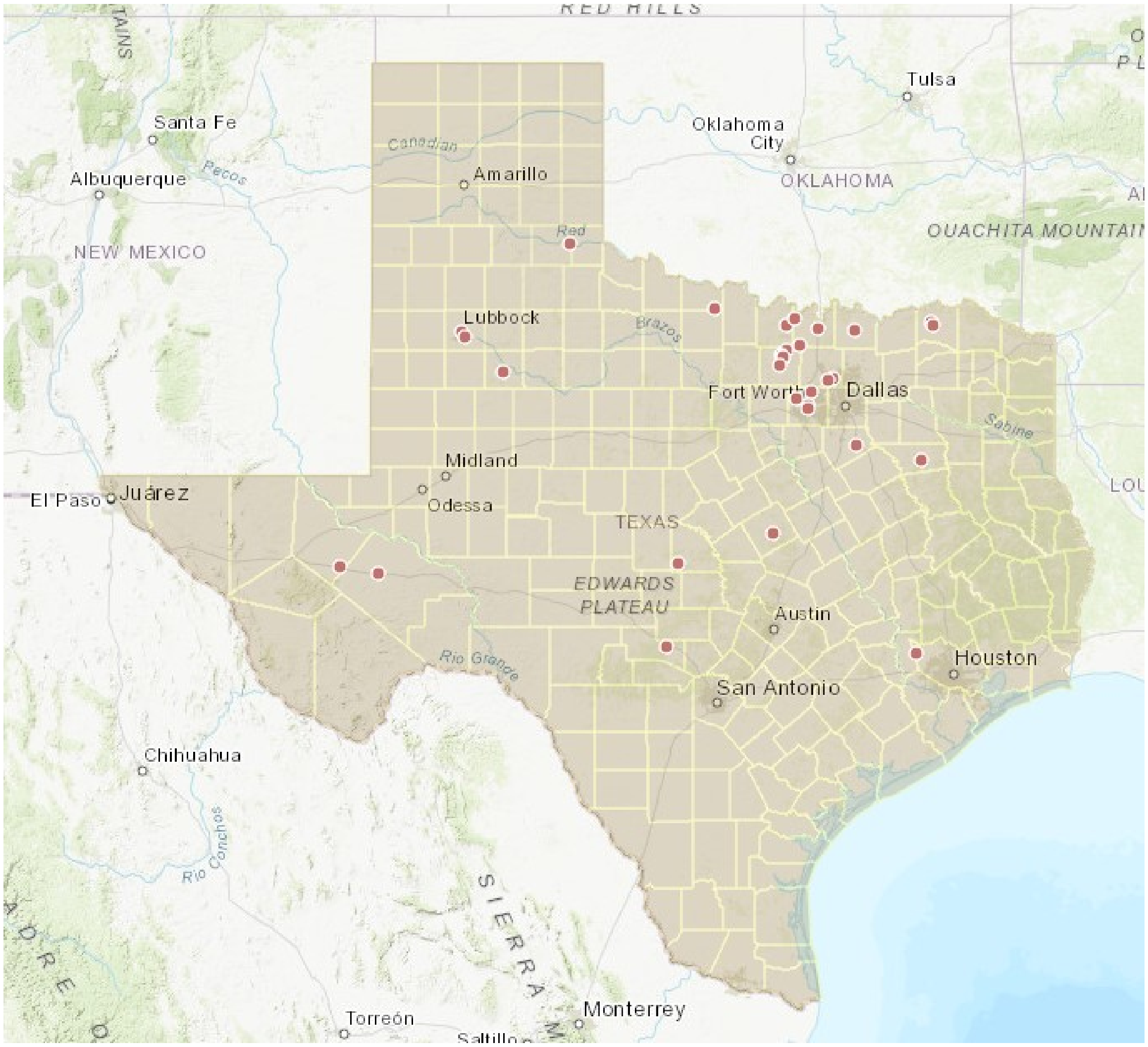
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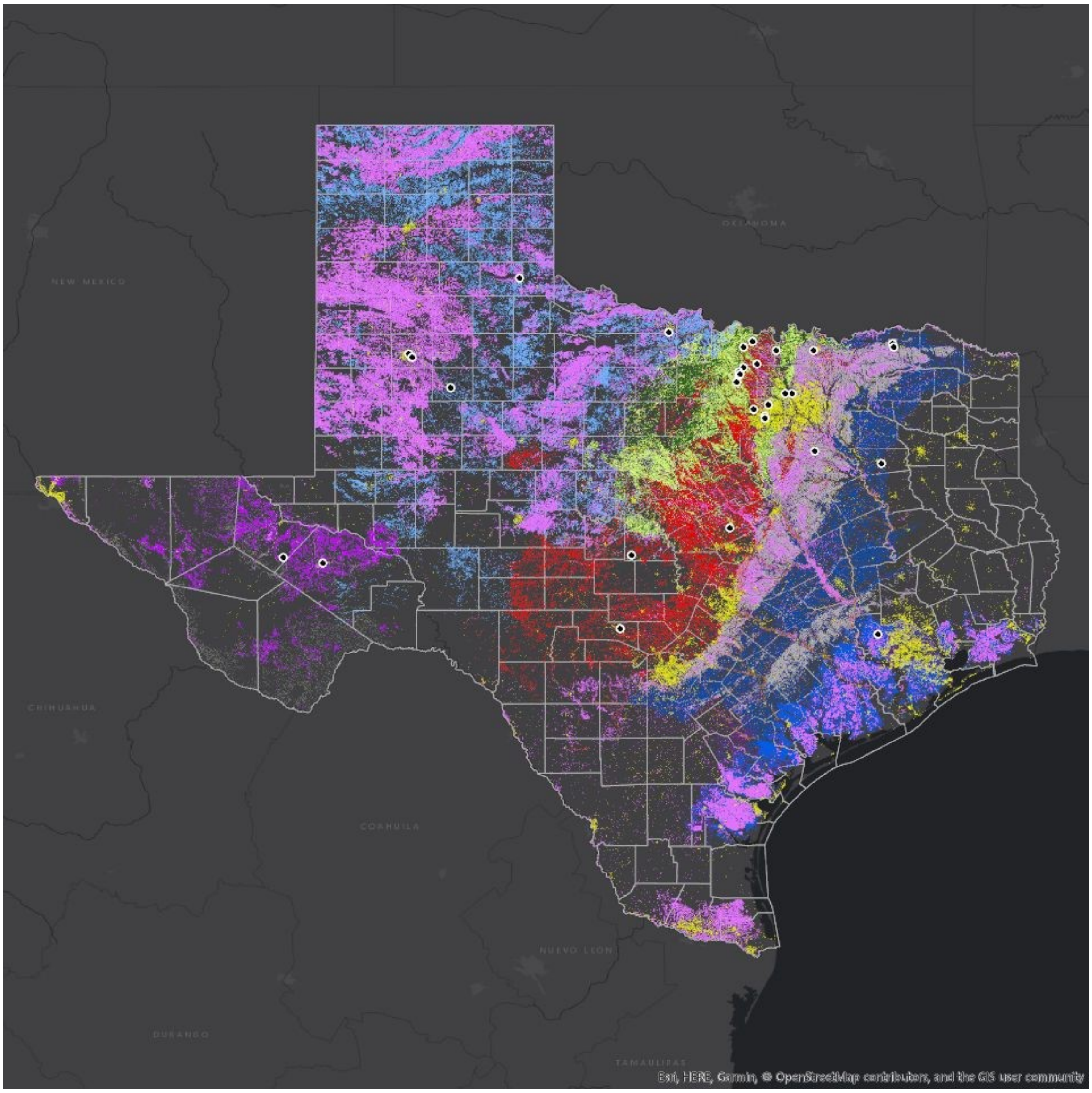
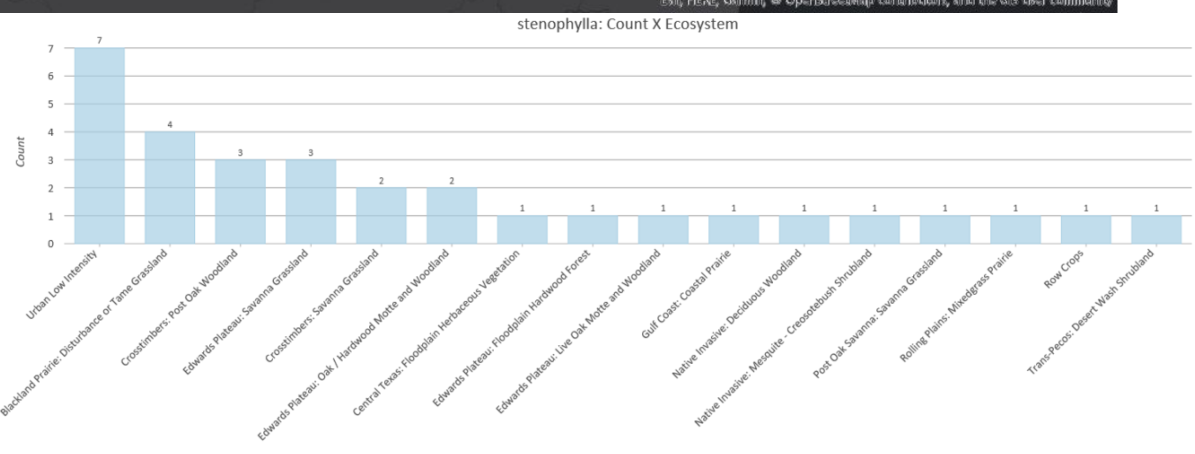
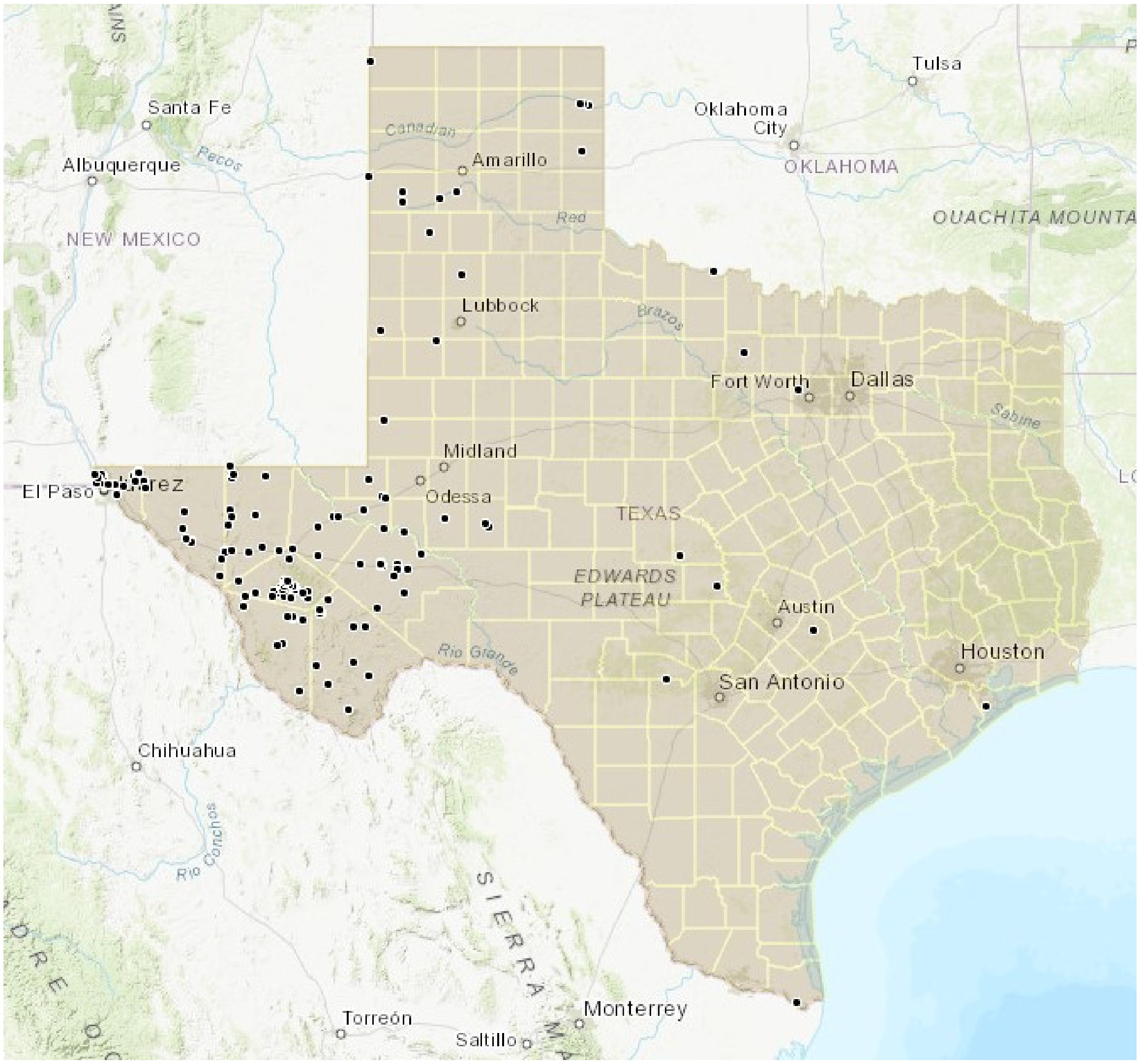




Asclepias subverticillata



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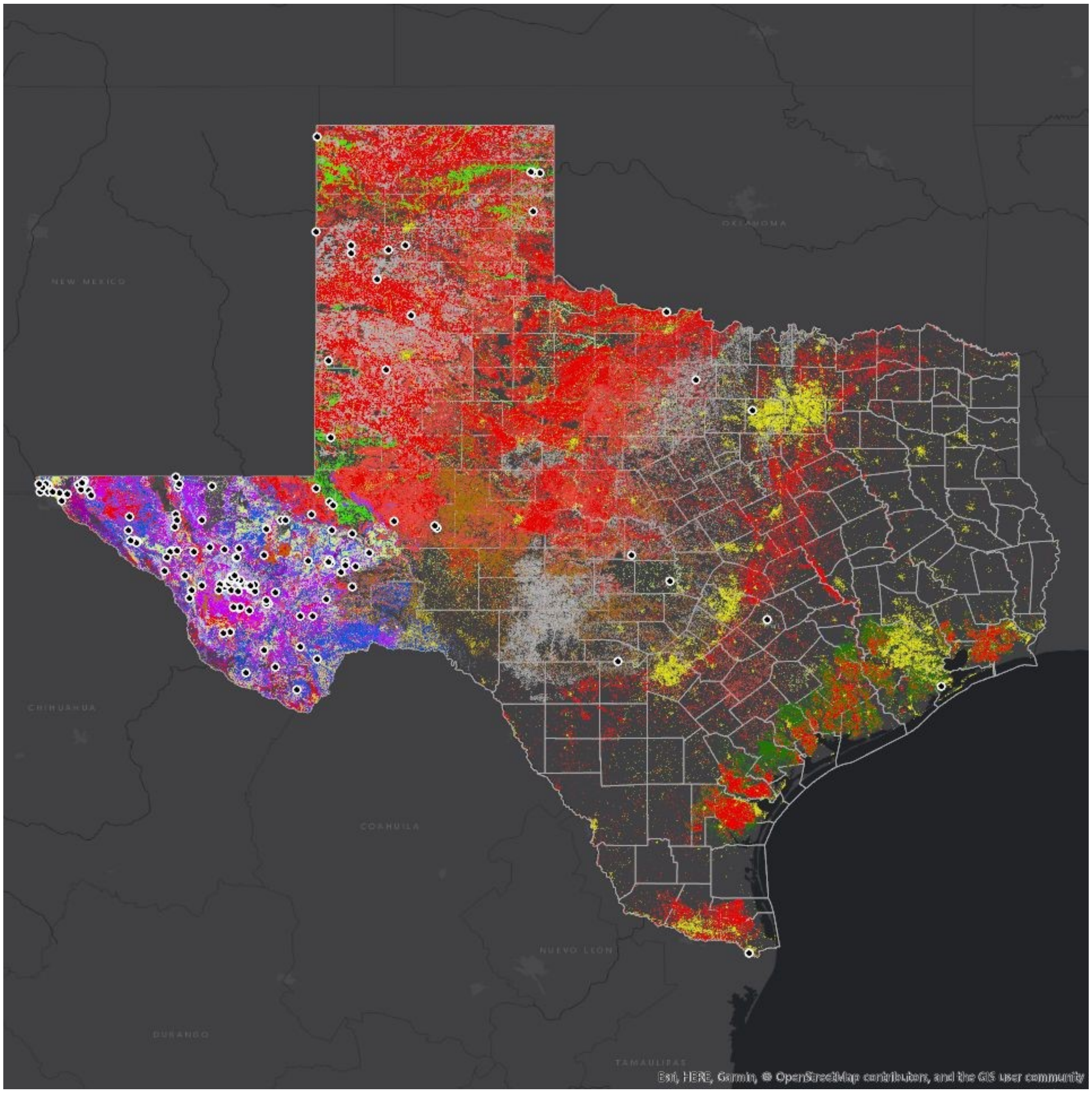
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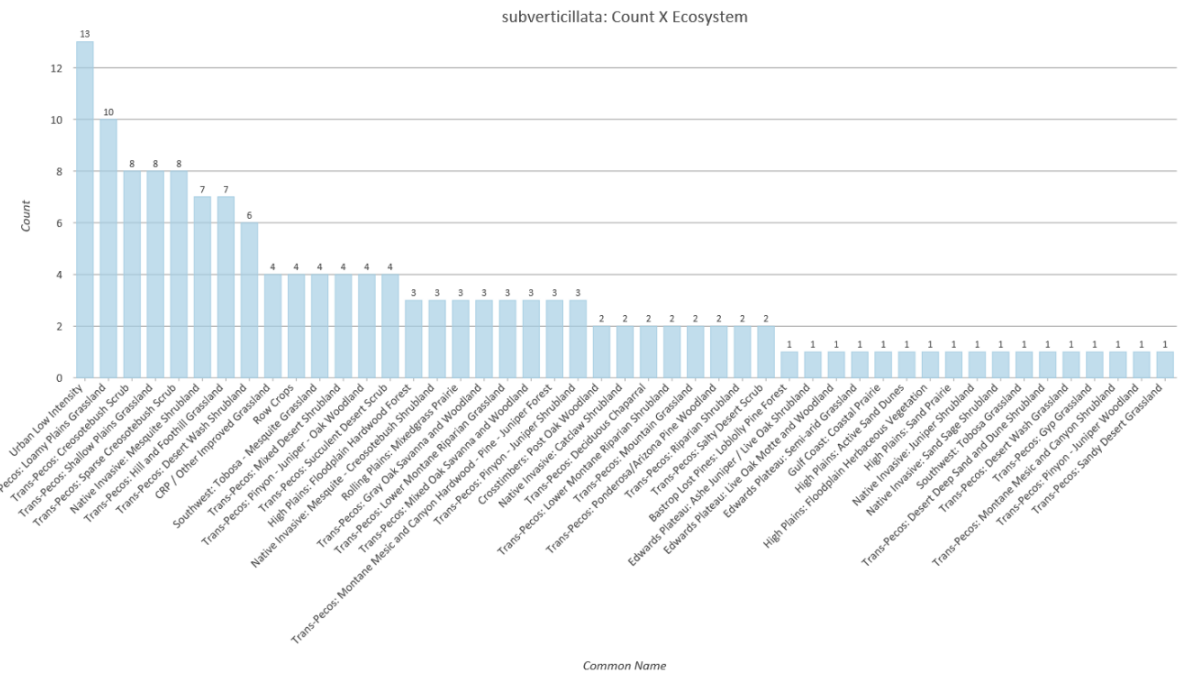
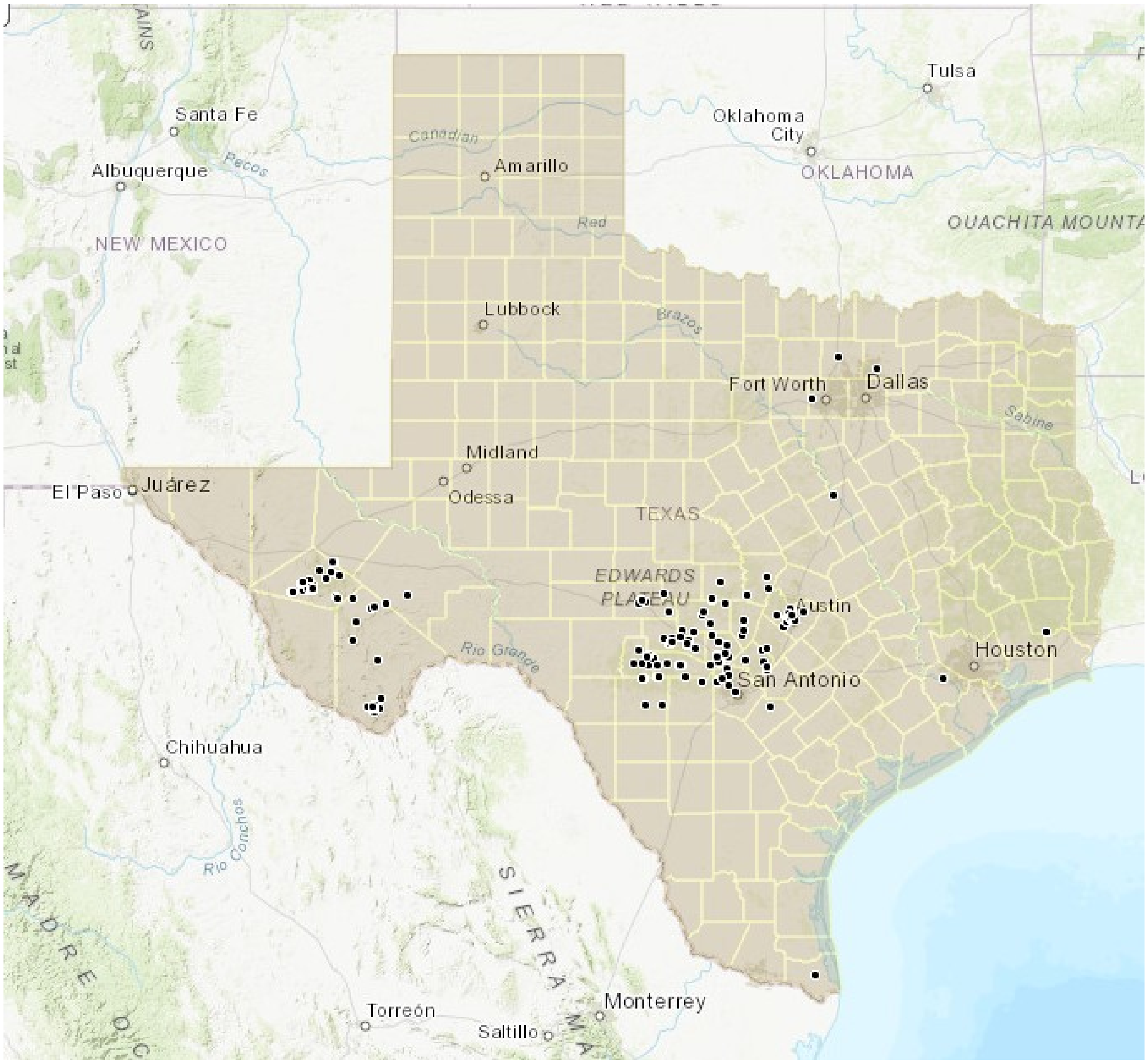


Asclepias texana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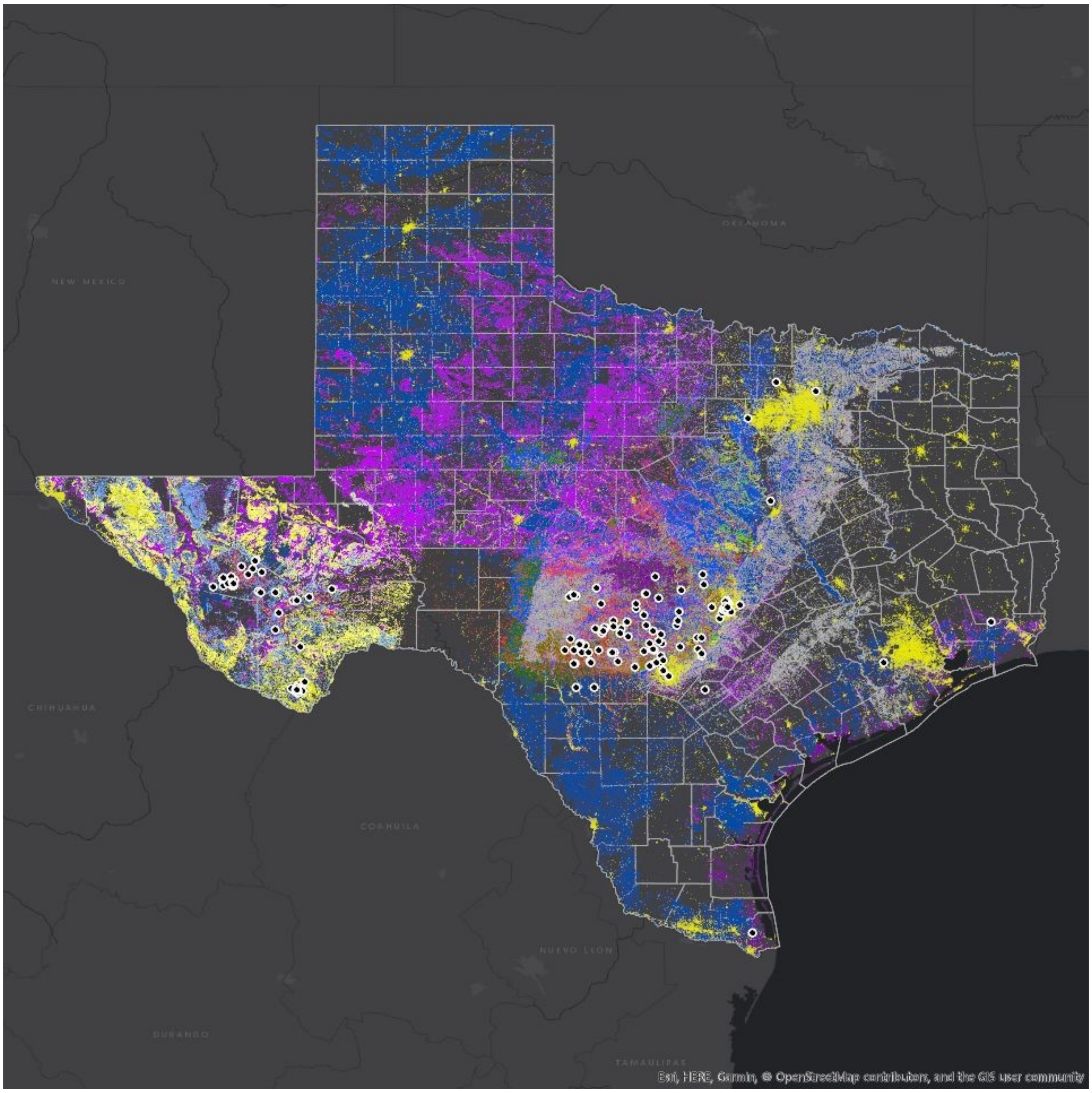
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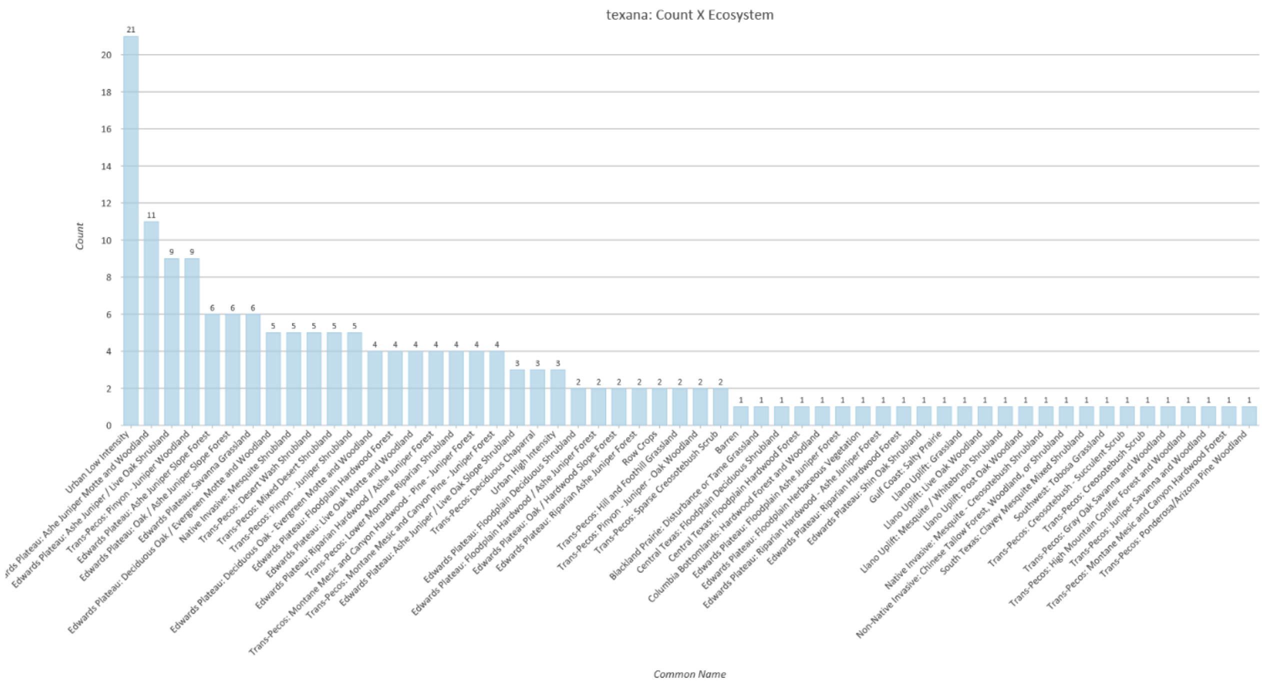
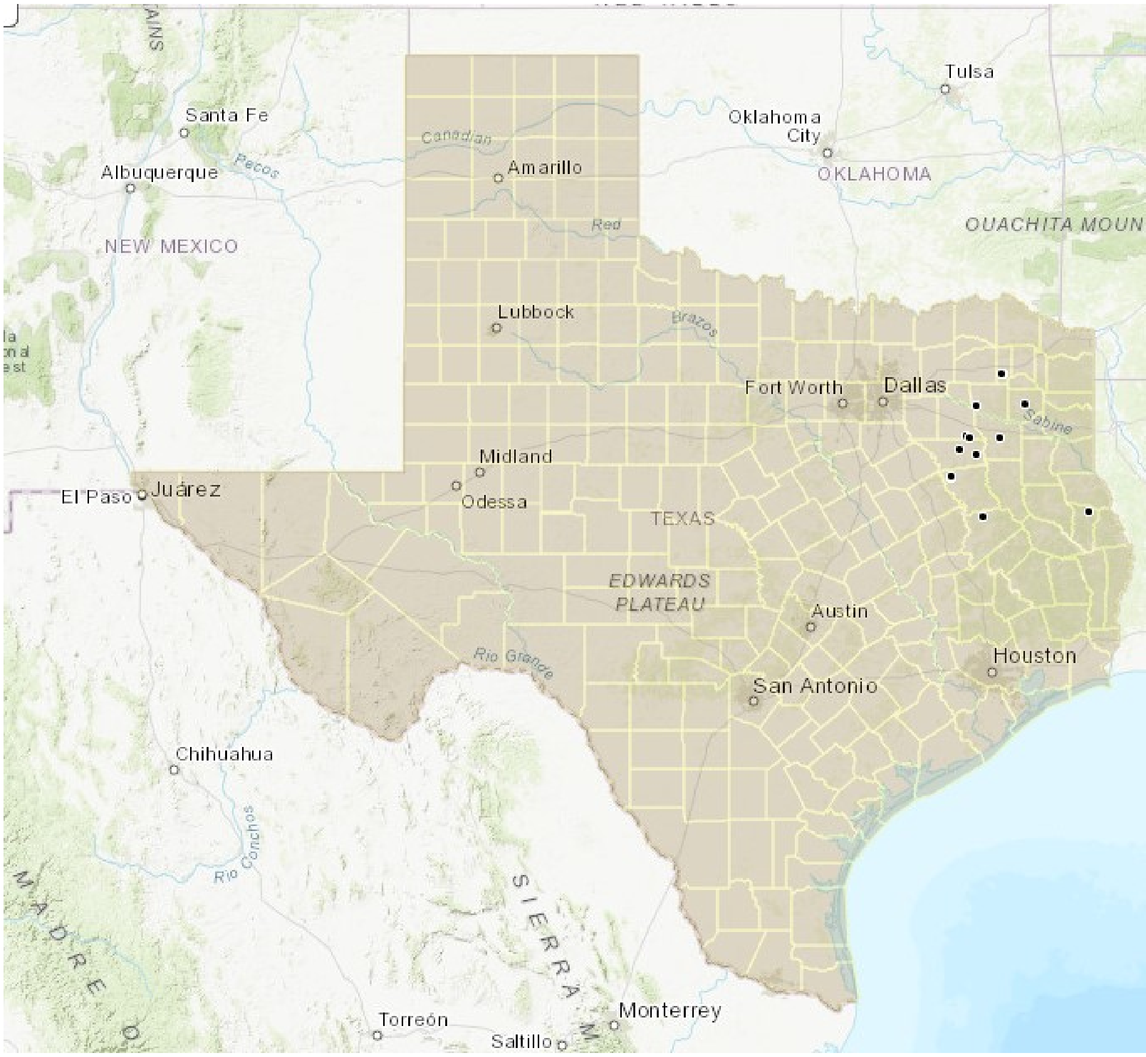


Asclepias tomentosa



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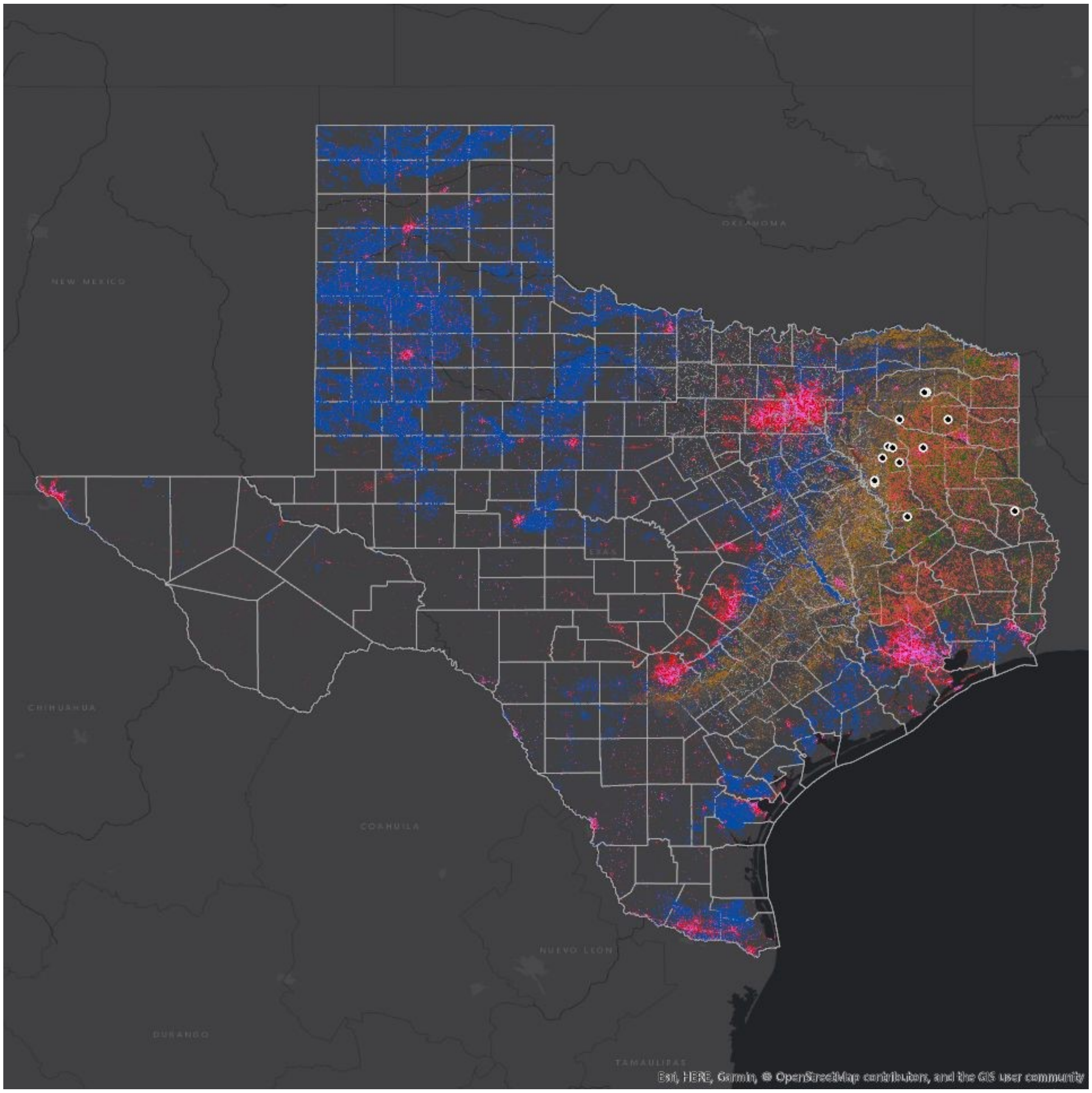
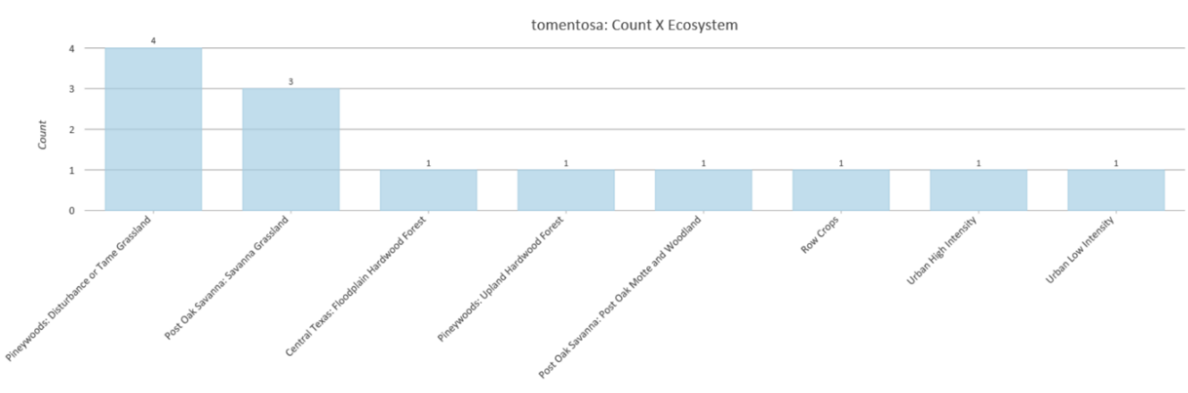
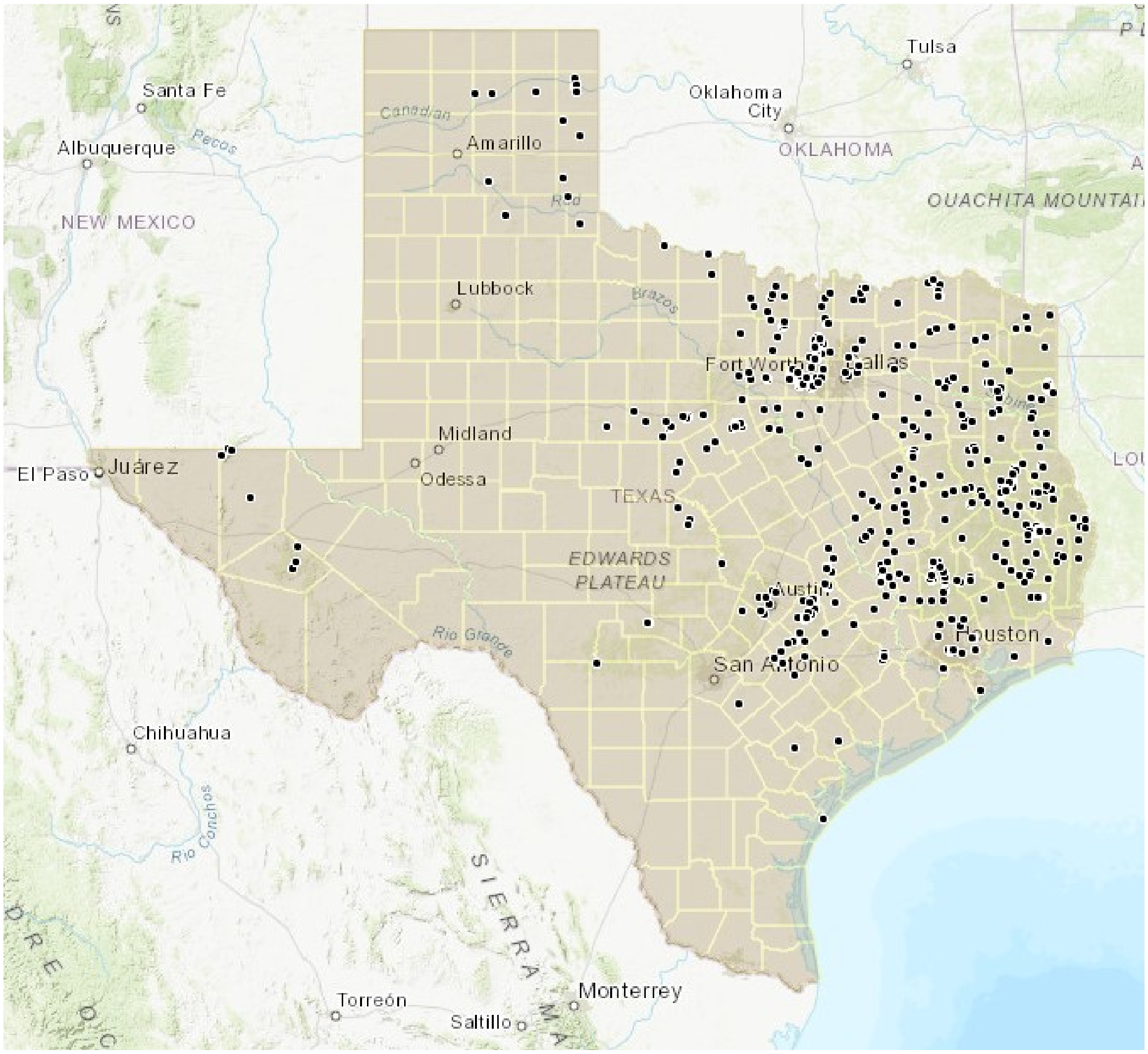




Asclepias tuberosa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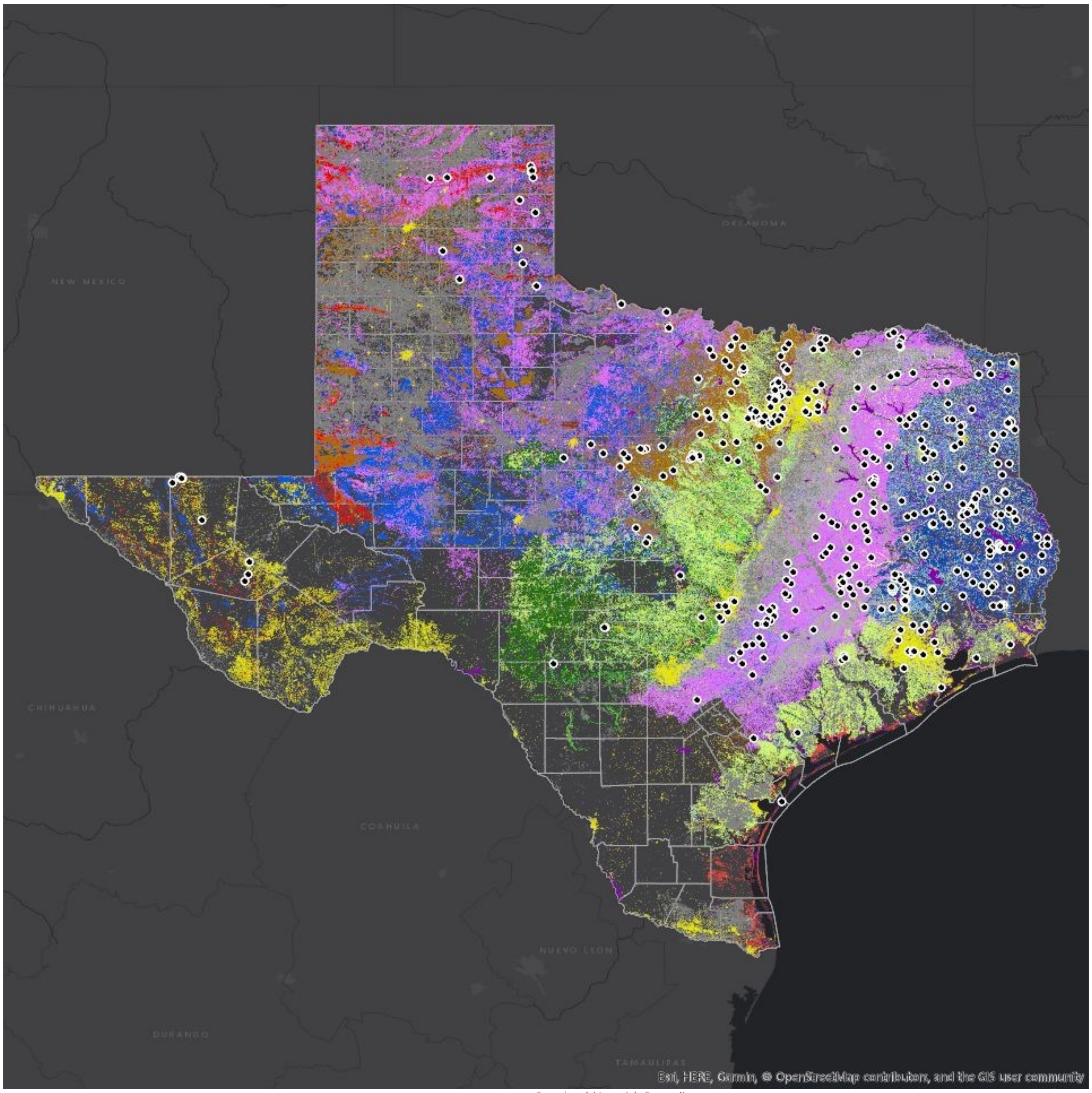
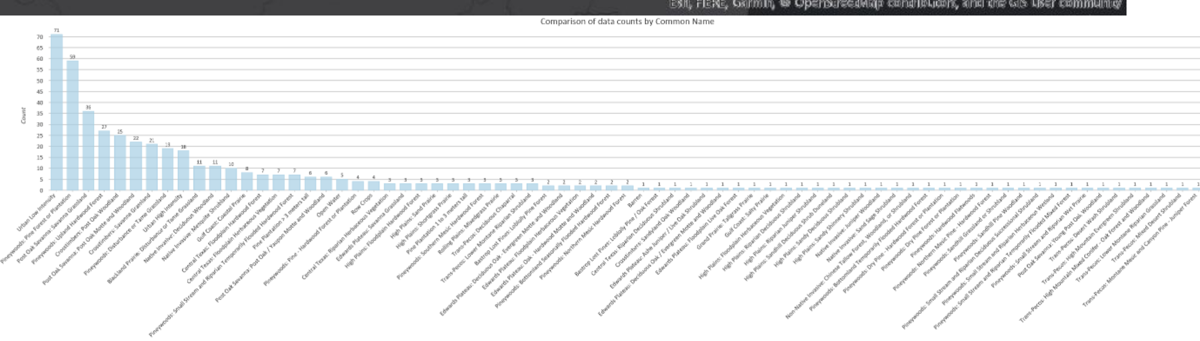
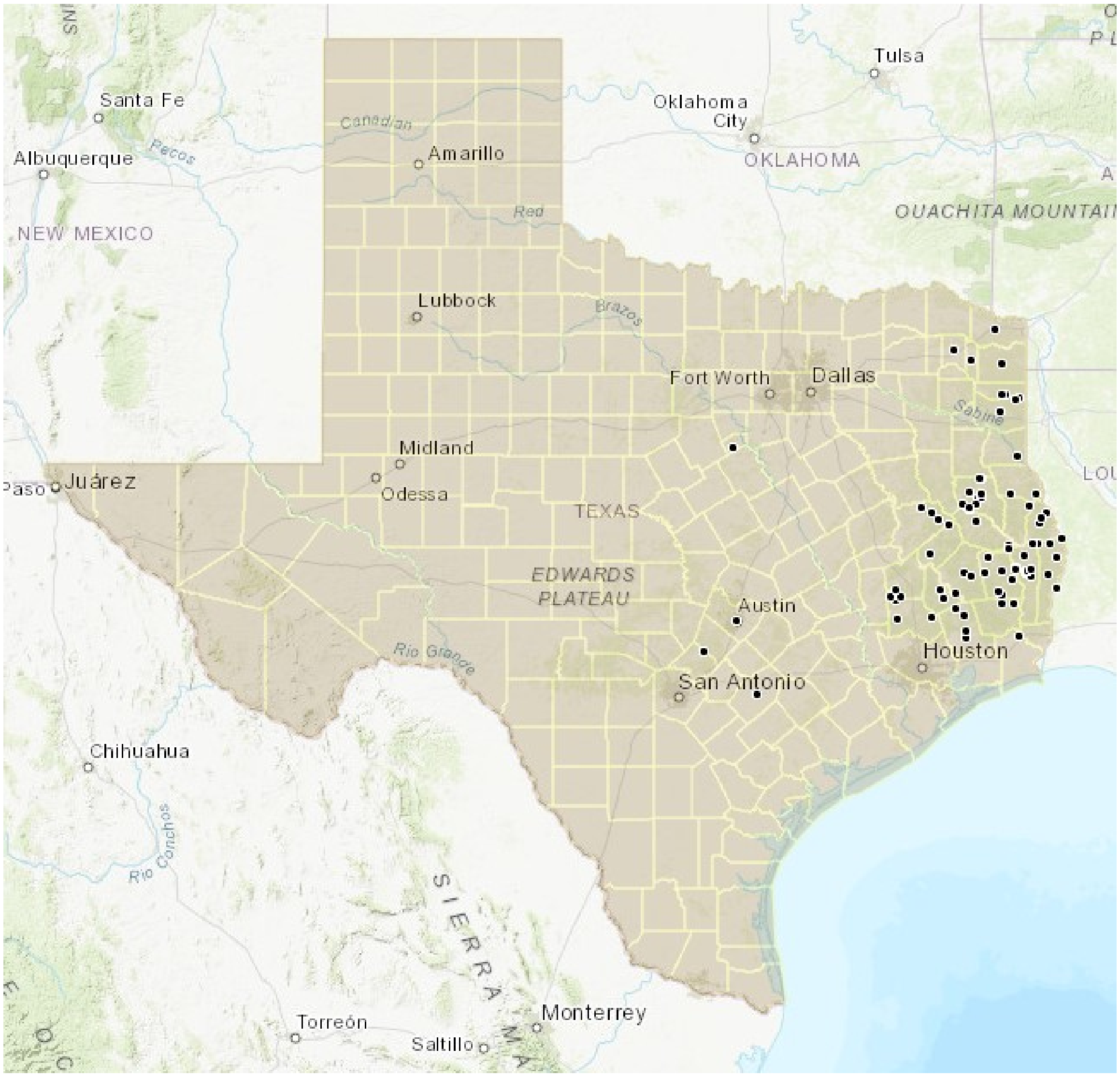




Asclepias uncialis eliminated from Tx list

Asclepias variegata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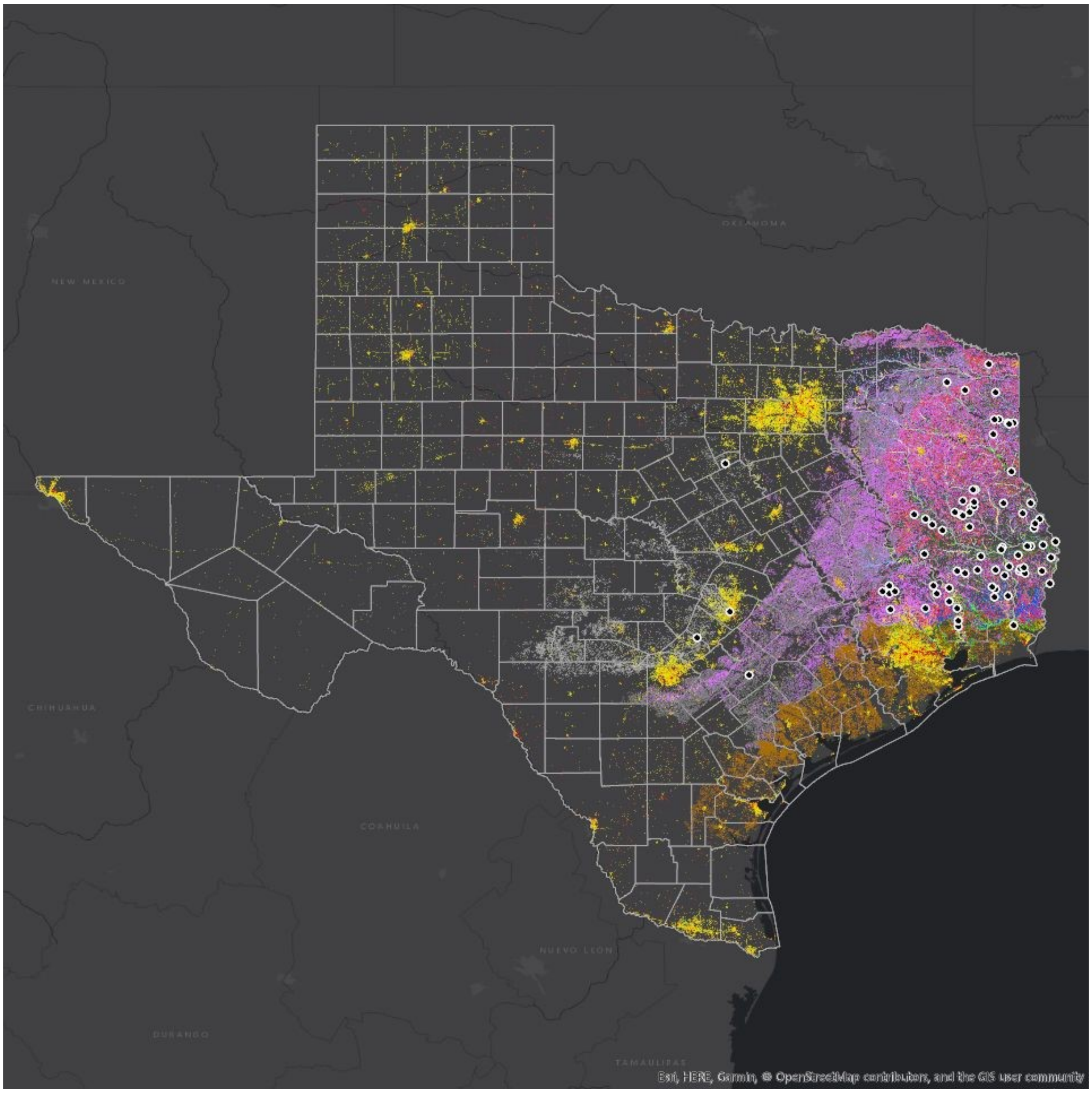
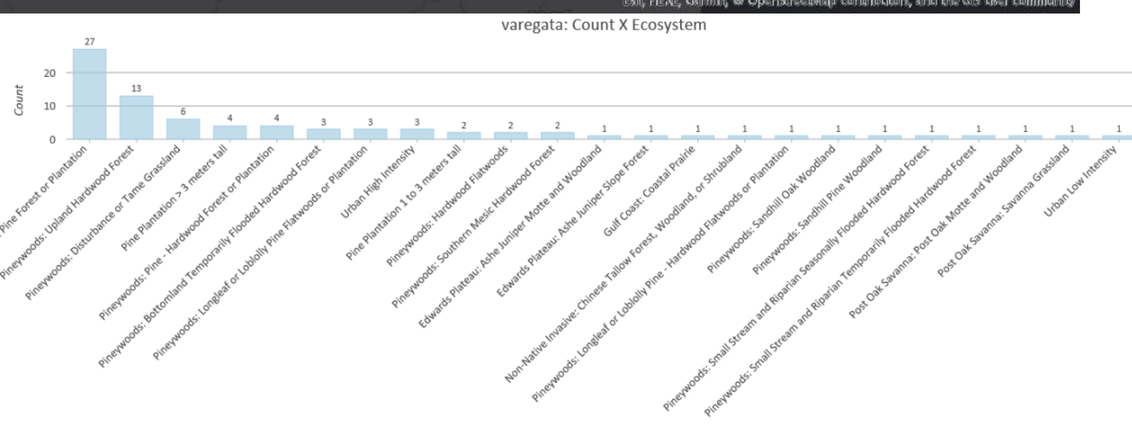
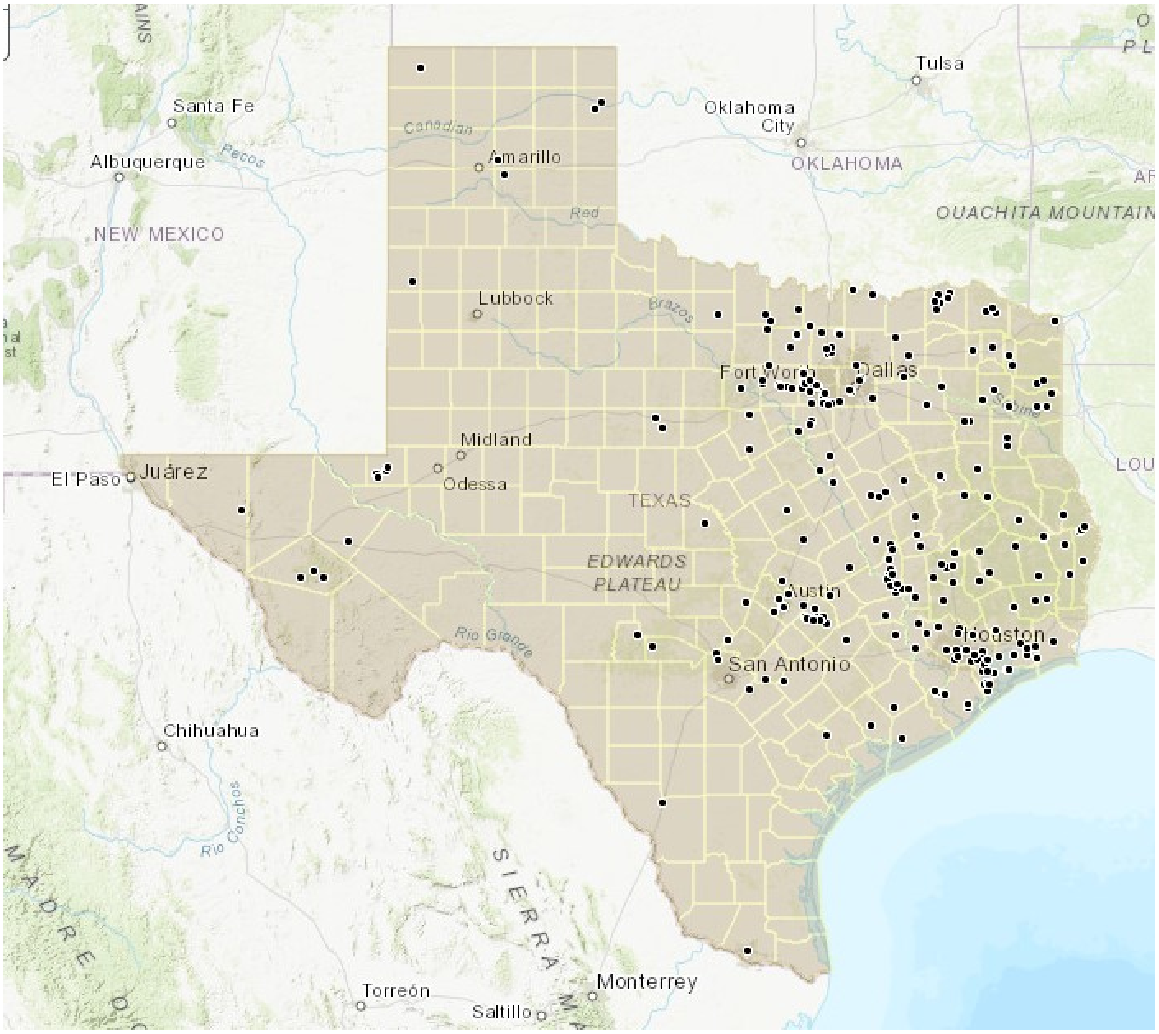




Asclepias verticillata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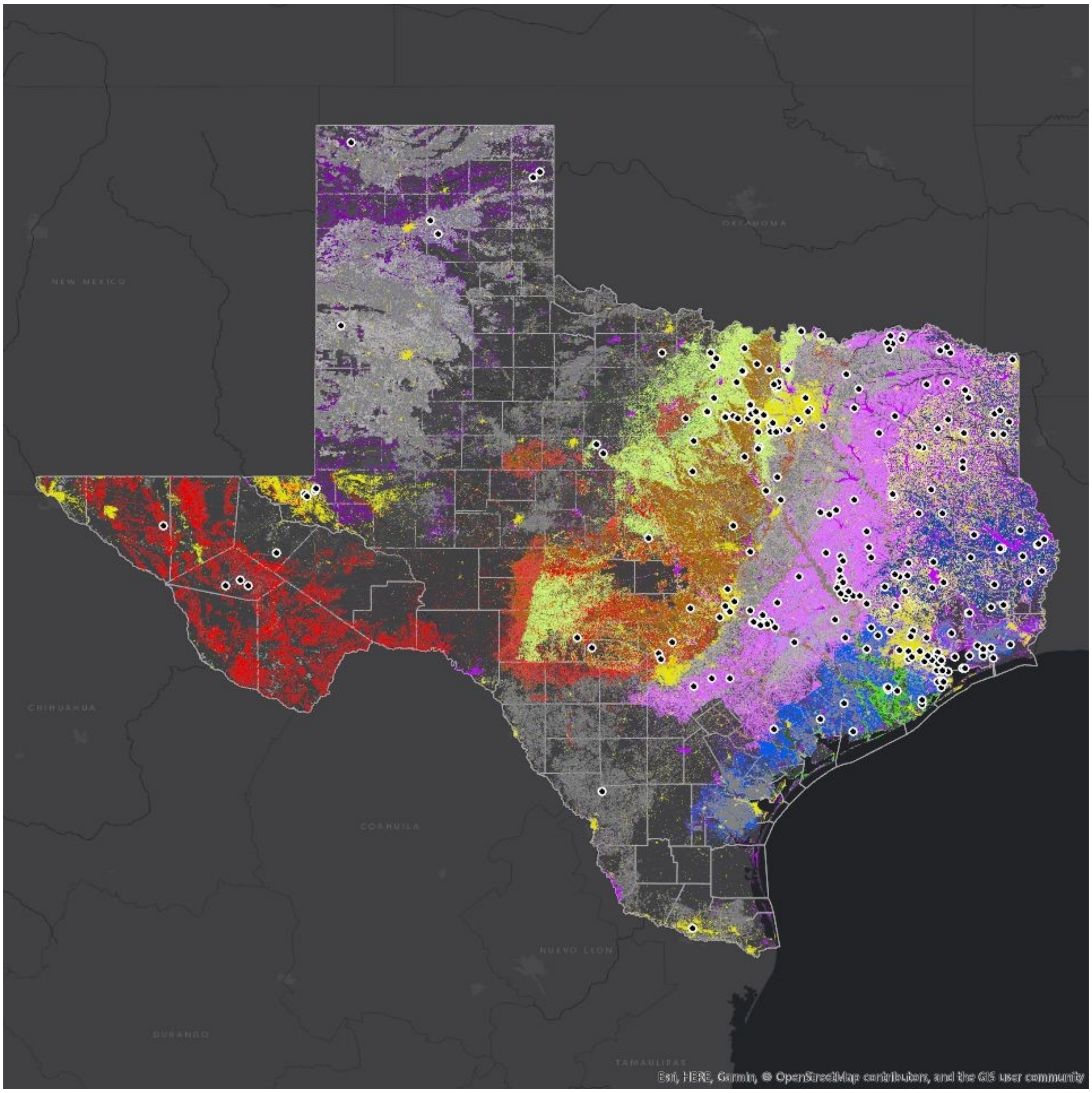
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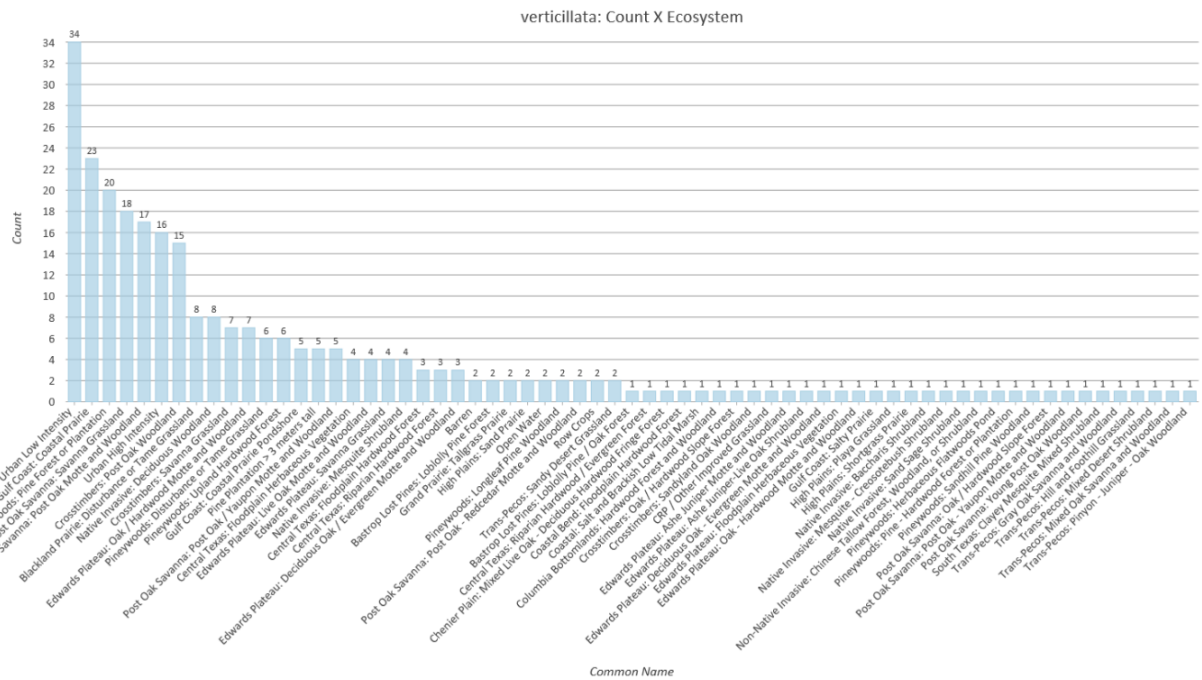
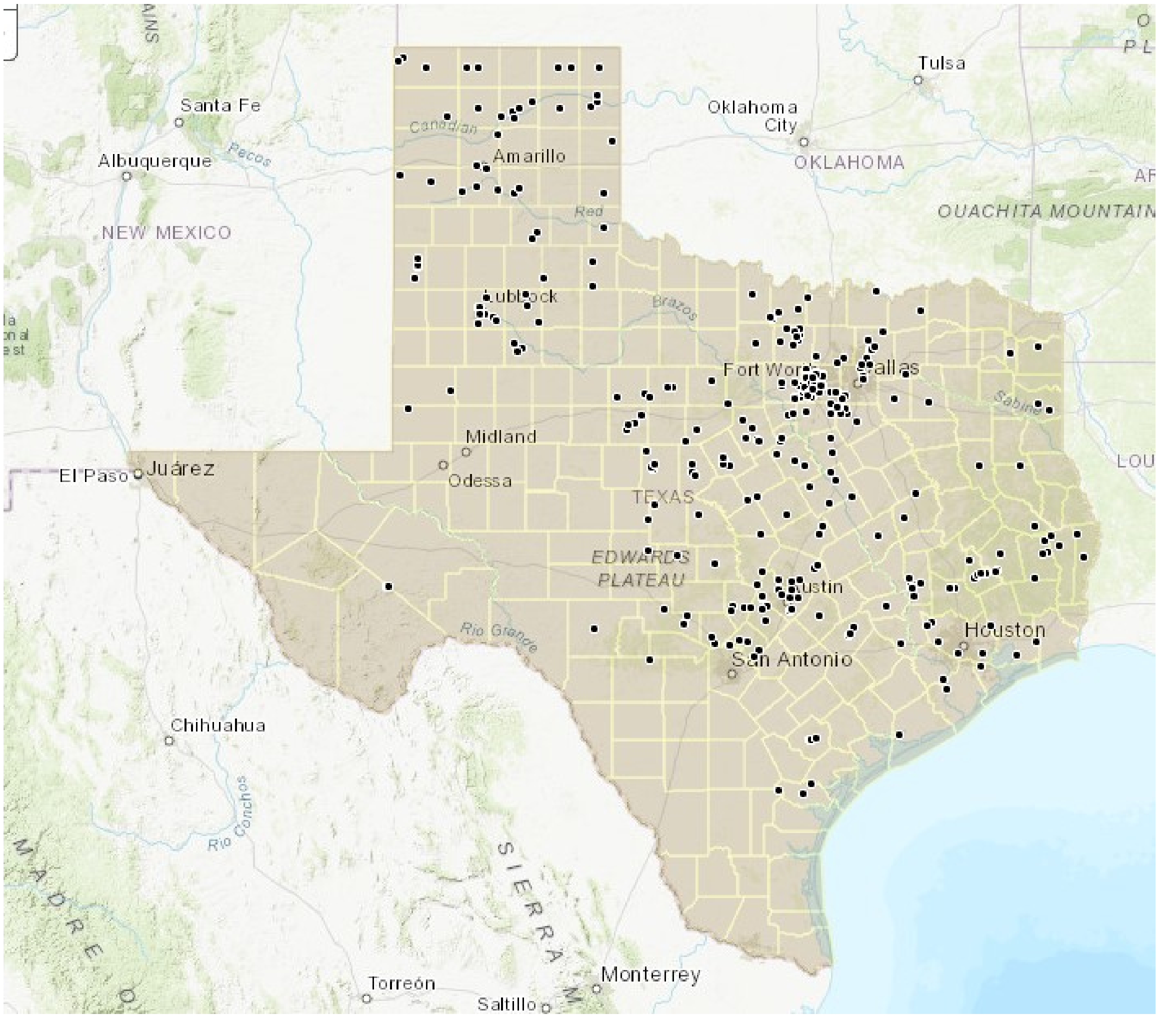


Asclepias viridiflora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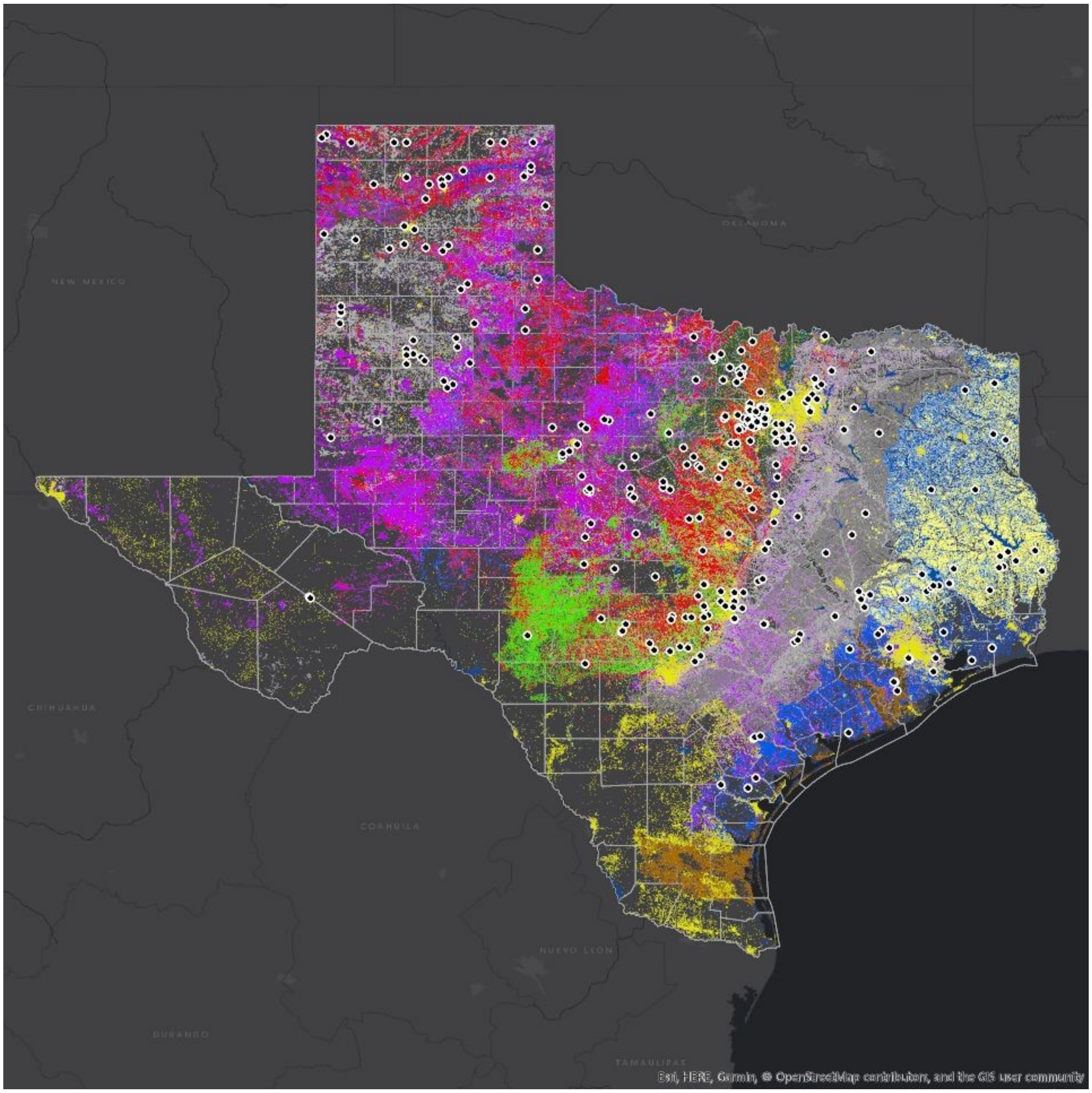
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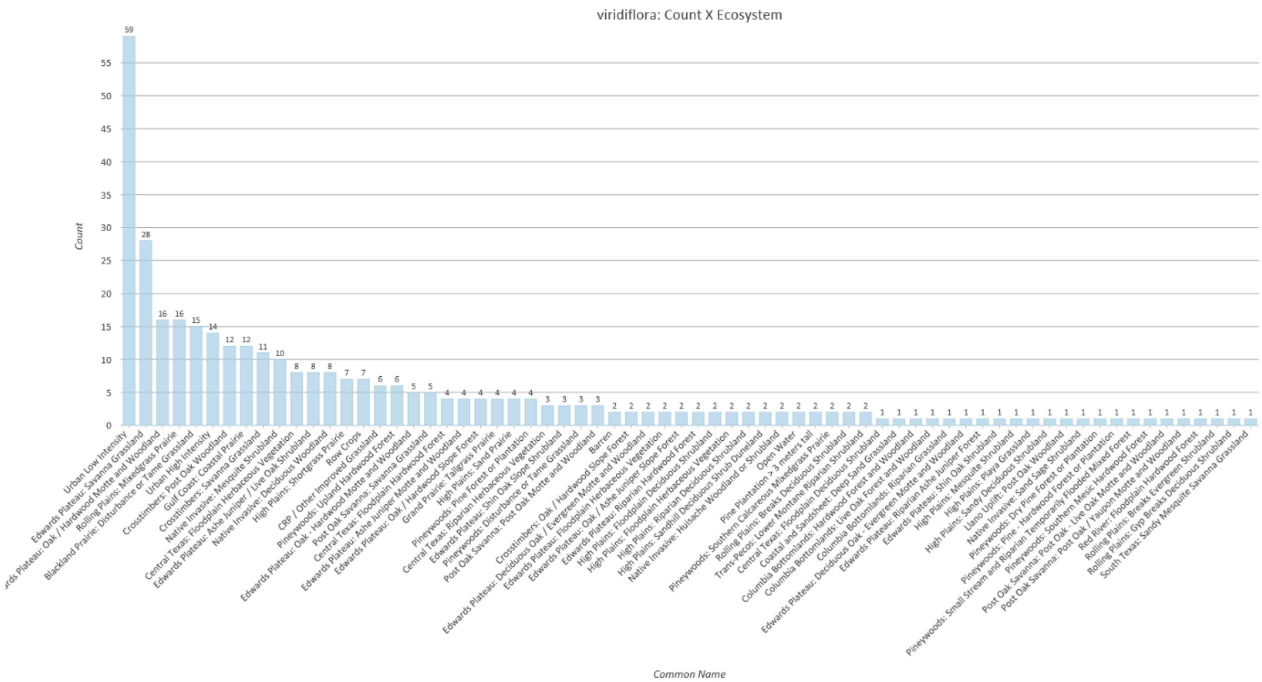
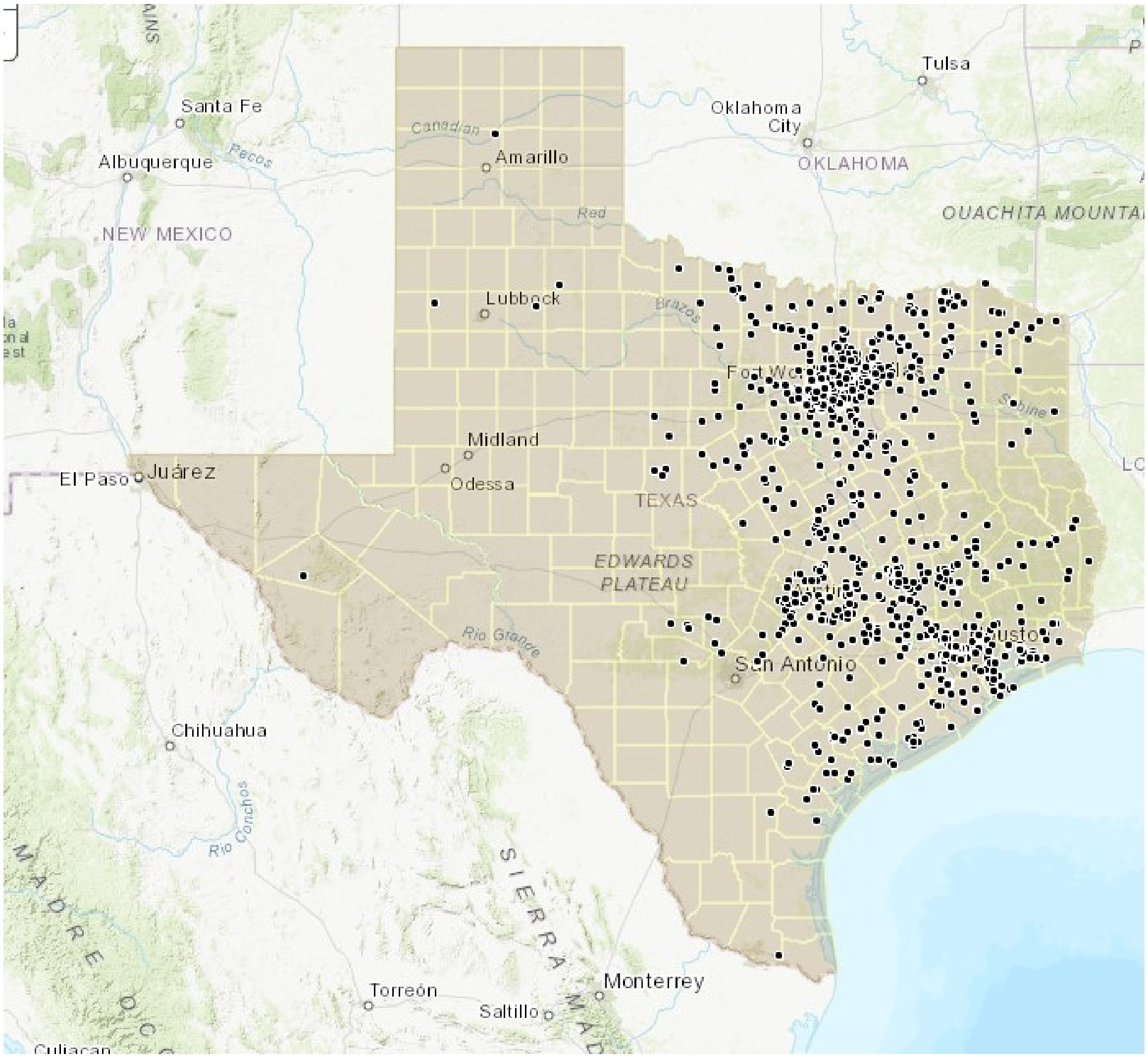


Asclepias viridis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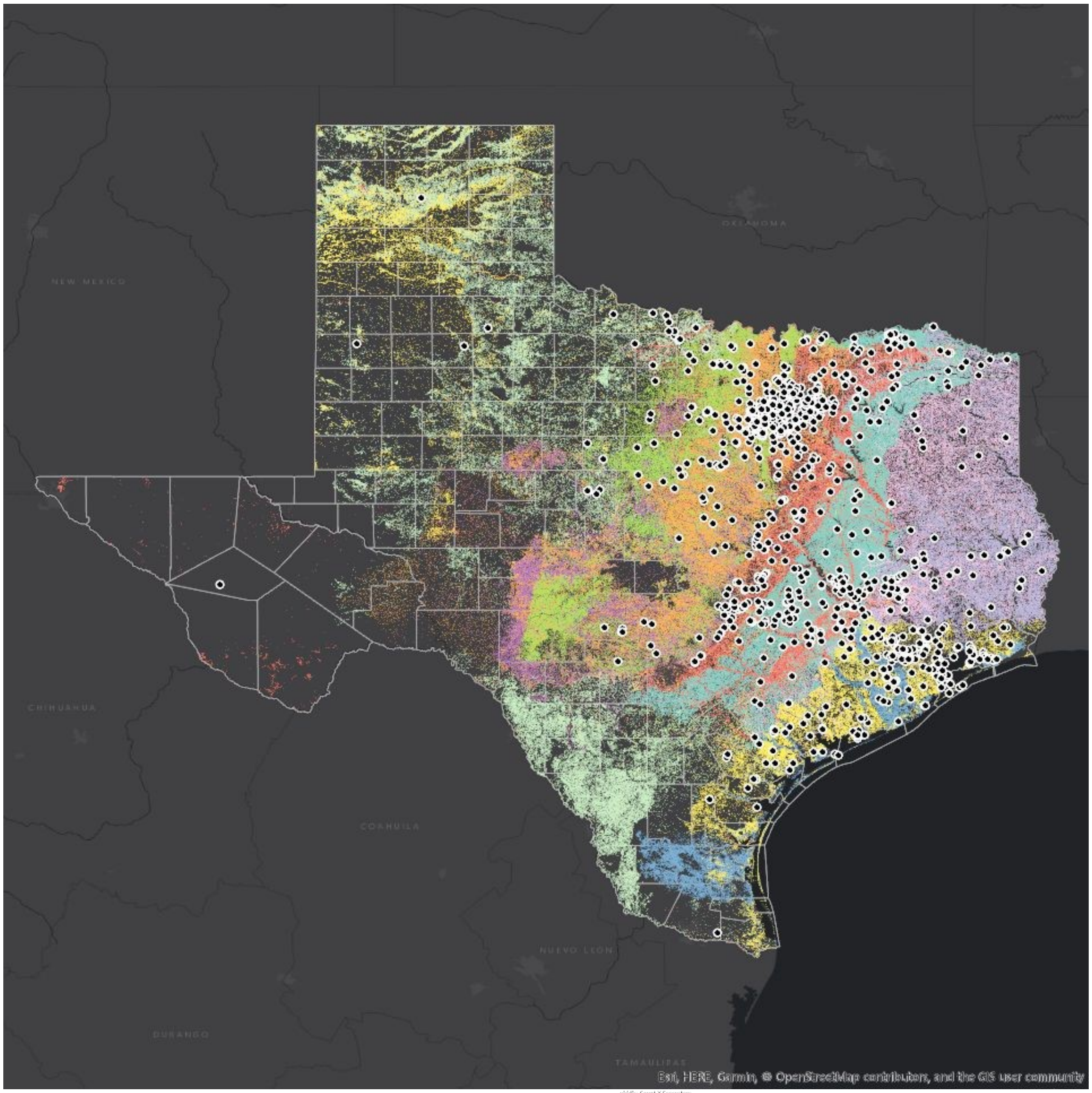
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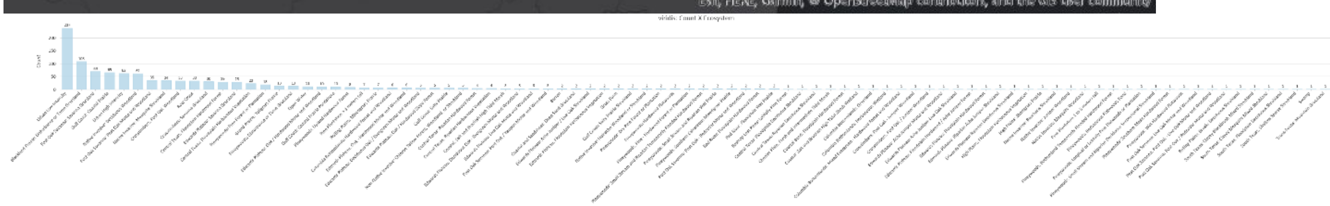


6. Value added to project: Mowing, milkweed availability, and monarch butterflies

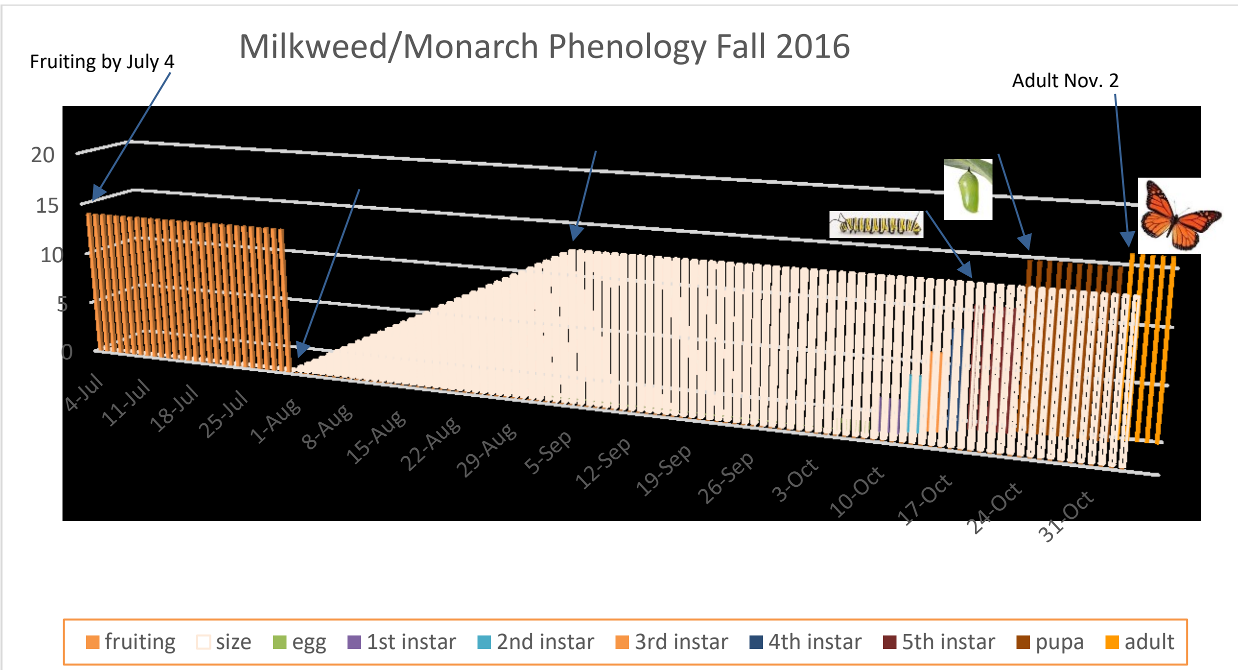
Timing of Mowing

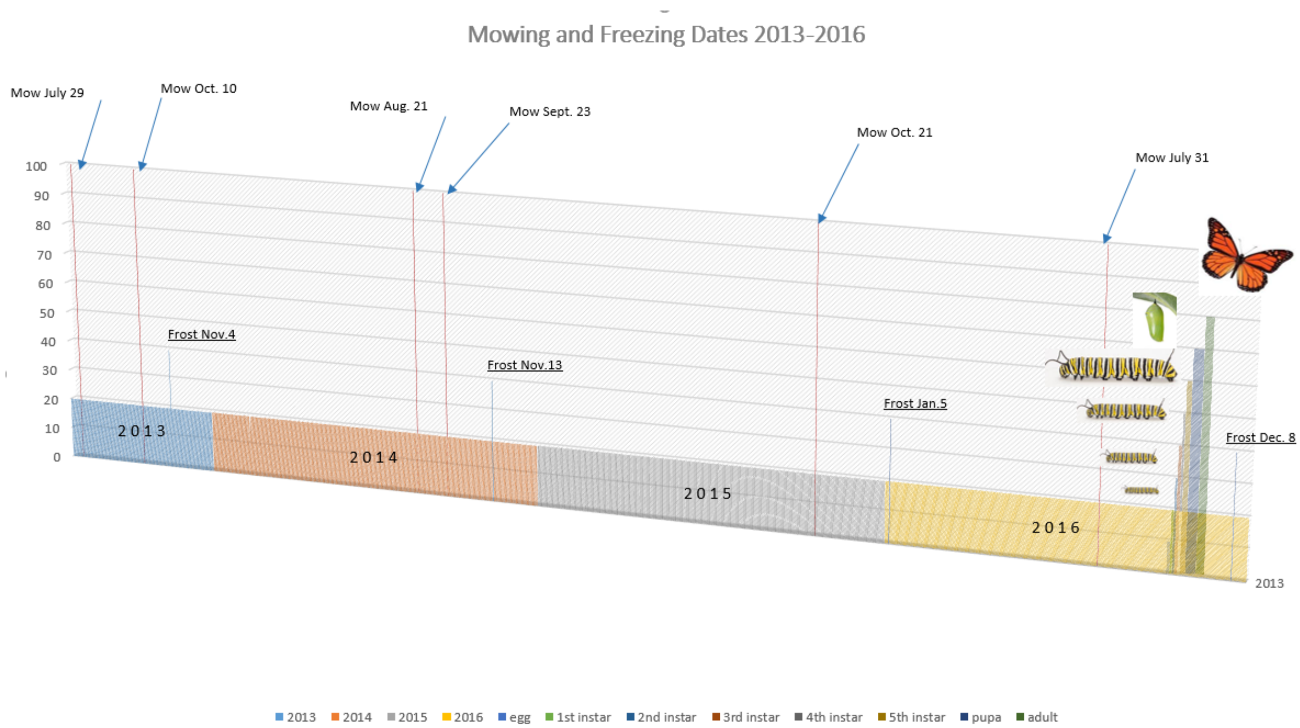
We have begun to collect data that shows the phenology of milkweeds must be understood to time mowing for monarch reproduction. This data will be important for making recommendations to private landowners and TxDot contractors who are mowing roadsides It is becoming apparent that the timing of mowing is a critical factor in increasing the milkweed density. It may be possible that minor adjustments to mowing schedules could produce a considerable documentation of action producing monarch increase.

An example of this type of consideration is the Godwin Farm in Wood County, Texas that has been in hay production since 1989. Since 2013 it has been on an unusual haying schedule with one mid-summer cutting and one late fall cutting. This property is shown below where there are Locations of 11 *Asclepias amplexicaulis* on 100-acre tract of Coastal Bermuda grass/bahaia grass pasture. The red line = 710 meters.

Example 1: Fall Reproduction at Milkweed #1 in 2016

All pastures were mowed and baled by July 31, 2016. By September 5 Milkweed #1 grew back in one stem approximately 14 inches high from the root. In the following 11 weeks the plant regrew, was oviposited on by a monarch butterfly and supported one larva to 5th instar. The 5th instar was discovered on October 18. It pupated on Oct 23 and emerged as adult on November 2. This also gives evidence for a "fifth generation" of monarch butterflies. Below is a representation of this data:





Above shows the History of hay cutting since 2013 on one site. This shows that haying in late July allows time for *Asclepias amplexicaulis* to re-grow to a size sufficient to feed a generation of Monarchs before the first frost. In fact, on *A. amplexicaulis*, observations in 2016 showed that plants were beginning to turn yellow and drop leaves by mid-October (above figure). It appears that in this case, larval development must reach 5th instar by mid-October. The pattern of haying in 2015 would have not favored this generation. They would have been mowed down as 4th or 5th instar larvae. In 2014 these meadows would have been mowed too late to allow sufficient re-growth of the milkweeds. In 2013, the haying on July 29 would have allowed the re-growth, but haying again on Oct. 10 would have destroyed eggs or early instar larvae. So we see that over the last four years of this hay meadow, it is likely that only one in four years would have produced a fall-generation.

Importance of Roadside Populations

In 2016 we identified numerous roadside milkweed populations. These populations were evaluated for species composition and density and are included in our section on milkweed distribution. We have determined that there is a distinct difference in plant density occurring between roadside and adjacent fields across the fence. All roadside data should be accompanied by other data on the paired density across the fence. This data will be critical to forming any management recommendations for roadsides. It appears that timing of mowing serendipitously selects for denser milkweed populations on roadsides. This appears favorable to Monarch reproduction in the Fall. But mowing in October is erasing this gain by destroying a large part of that generation. In effect the roadsides appear to be operating as traps to absorb fall reproduction.

Milkweed Phenology

It has become apparent that milkweed development and timing data will be critical to management for Monarch Butterflies if needed. We need to collect data that shows for each species; when in spring does each species begin to grow; How long is required for re-growth after mowing; when is flowering/fruiting relative to first growth; Do plants senesce in Fall or persist until frost. This could be an important future study if conservation of the monarch butterfly is needed.

7. Value added: Monitoring of Milkweed Availability for Monarch Butterflies

Data collection has progressed for monitoring milkweed populations. Host plants are being evaluated for availability (phenology) for monarch butterfly reproduction and actual usage of those milkweed populations. We have attempted to locate populations of as many Texas milkweeds as possible. Our research has resulted in major changes to the list of the Texas species. Two species may be removed from the list. One will probably be added. There are 36 species of milkweed reported for Texas but our research is showing that the number is now 34. Of these 34, we have observed 29 of these species in the field and recorded their locations. We are in the process of mapping these species for the entire state. We hope to locate the other five species by the end of our project.

<u>Changing list of Texas Milkweeds</u>

Asclepias purpurescens was formerly listed as a Texas species based on collections in Bowie County in 1958. Our searches for this species have been fruitless. This rare species will probably be de-listed as occurring in Texas.

<u>Asclepias uncialis</u> is a Rocky Mountain species that was formerly known from the vicinity of Andrews, Texas. This specimen has been identified as actually a small specimen of *Asclepias brachystephana*. *Asclepias uncialis* will be de-listed as occurring in Texas.

Asclepias hirtella occurs in a rare type of native prairie in NE Texas. This species has been lumped with *Asclepias longifolia* since 2009, but this species will probably be elevated again in the near future.

<u>Monitoring Urban Populations on Tropical Milkweed</u>

Observations of Monarchs in urban gardens in the Houston area have continued at a constant rate. Partners are texting or emailing observations and other data is coming in from iNaturalist. These data are being compiled and will be included in a future report.

<u>Milkweeds and their Place in the Landscape</u>

It is becoming apparent that some milkweed species are common in the landscape and occur in dense populations while others are very rare, occurring in only a handful of populations and still others are common, but occur as widely scattered individuals. This information will be important in the final report for ranking milkweeds by their importance for monarch reproduction. Three styles of growth are highlighted in this report.

Style 1: *Asclepias viridis* is a common, abundant species of roadsides and pastures with cattle or horses.

Style 2: *Asclepias tuberosa* Figure and *Asclepias amplexicaulis* occur in widely scattered clumps across the landscape, often with many miles between populations.

Style 3: Species like *Asclepias rubra* or *Asclepias hirta* are very rare because of narrow habitat requirements, but can be locally common and dense. It is interesting to note that some species like *Asclepias amplexicaulis* occur in widely scattered populations, yet appear to host more larvae than common roadside species like *Asclepias viridis*. It is premature to draw any conclusions but by the end of the fall our data should allow some conclusions to be made on this question.

Style 1: Common, abundant in dense populations.

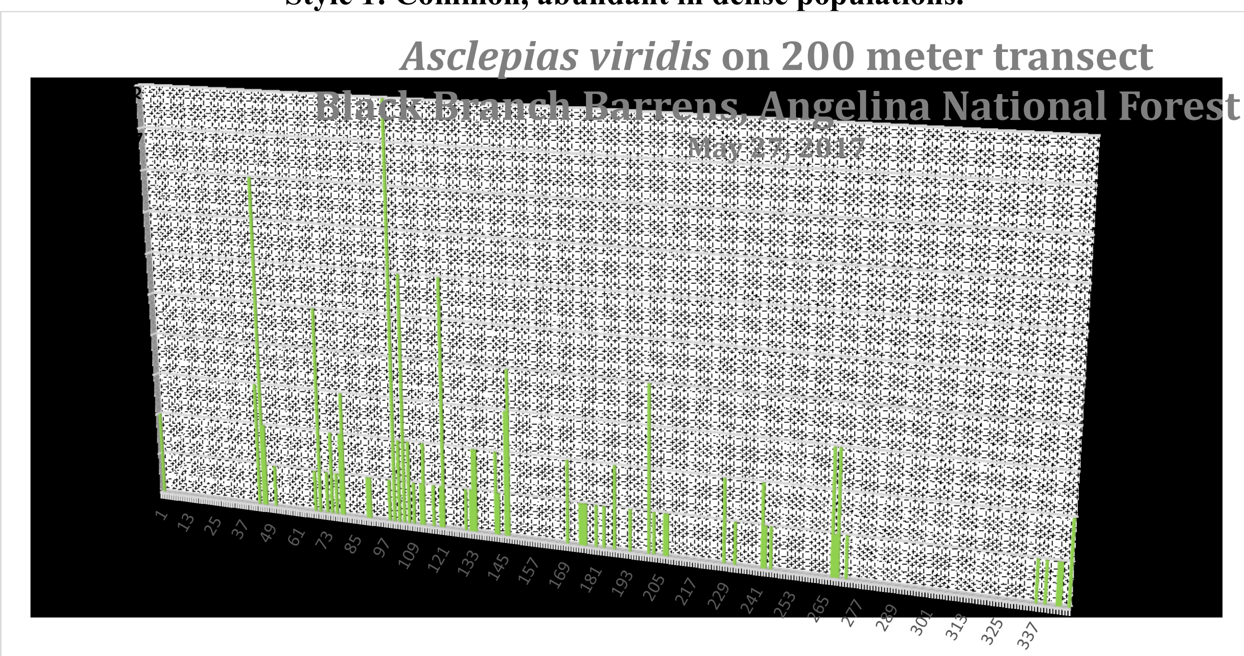


Figure 1: *Asclepias viridis* occured in dense, constant populations along the roadside of Highway 69 in the Angelina National Forest. But no larvae were present. Also noteworthy, the adjacent National Forest land with similar open areas in a site called

Black Branch Barrens has no Asclepias viridis occurring over a 600 meter transect through the natural area (Figure 4)

Figure 2: No *Asclepias viridis* were observed along a 600 meter transect through the natural area called Black Branch Barrens in the Angelina National Forest, but the adjacent roadside supported many hundreds of plants just a few feet away.

Style 2: Common, but widely scattered over the landscape.

Asclepias tuberosa occurs as widely scattered individual plants. Sometimes these single plants occur miles apart. One of the most dense populations we have located in Rusk County has plants or small clumps of plants occurring about a kilometer apart, yet this species has been the source of some of the rare sightings of spring Monarch reproduction (Figure 3).

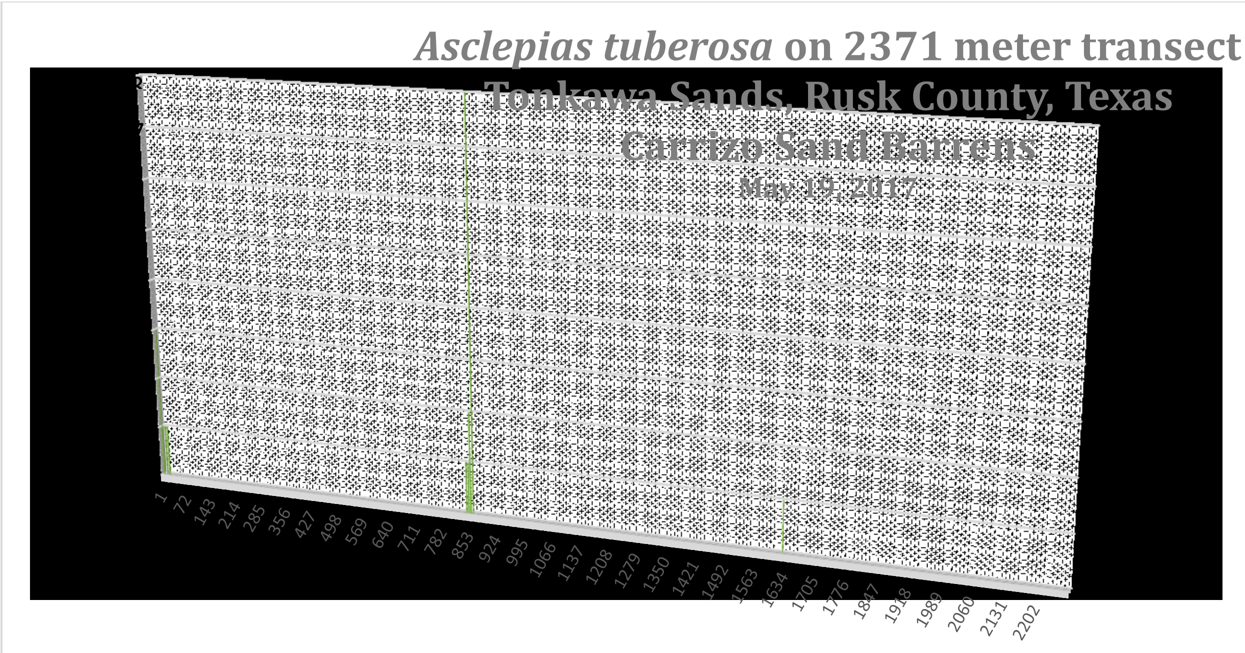


Figure 3: Monarchs reproducing on *Asclepias tuberosa*.

Style 3: Very rare because of narrow habitat

Several species of milkweeds are rare because of their narrow habitat requirements. These are mostly species of desert mountains, bogs and un-disturbed native prairies. *Asclepias hirtella* in one of these in prairies. It has been found to be dense at the Native prairies Association of Texas site named "Little Prairie" in Bowie County (Figure 4).

Asclepias rubra is another rare species that is limited to bogs in eastern Texas. We have searched for this species in 3 locations before finding a significant population. Soutendijk Bog in Wood County had a single plant. Boykin Springs bog in the Angelina National Forest had a single plant. The Geraldine Watson Rare native Plant Preserve in Hardin County has four plants that are being monitored by Pauline Singleton. Mr. Peter Loos and Mr. Joe Liggio guided us to a roadside bog in the Angelina National Forest that had the only significant population we have been able to locate(Figure 5 & 6).

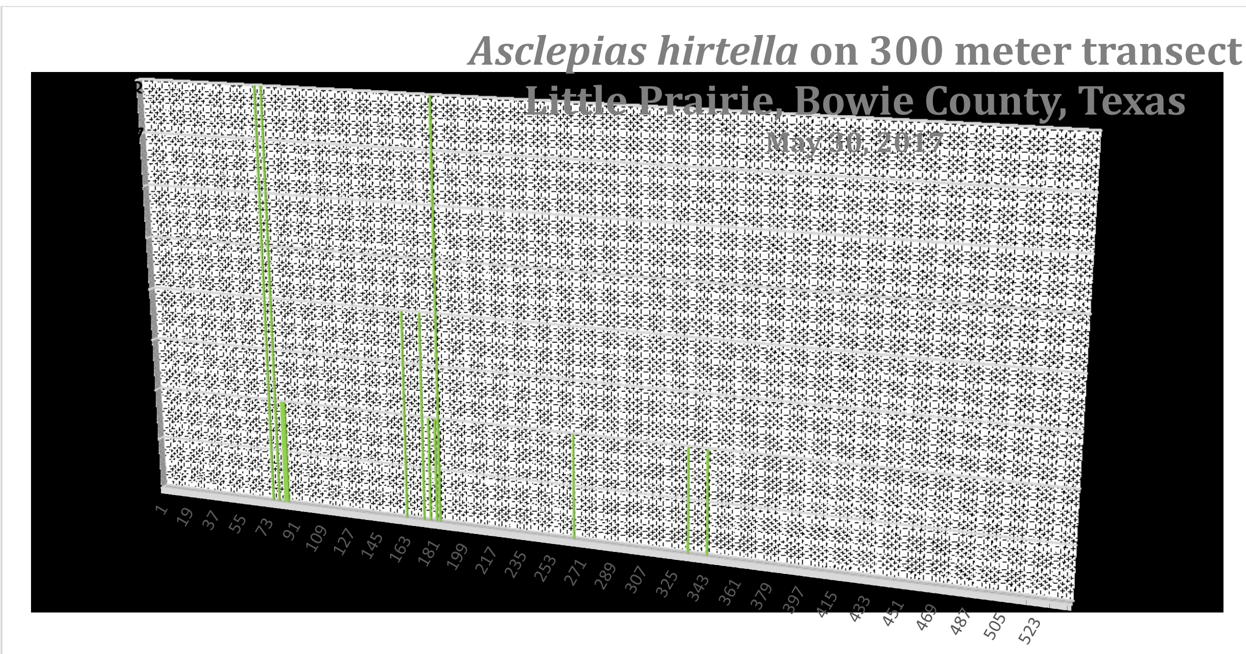


Figure 4: *Asclepias hirtella* is common with large gaps lacking plants on the Little Prairie.

Figure 5: *Asclepias rubra* in roadside bogs in the Angelina National Forest.

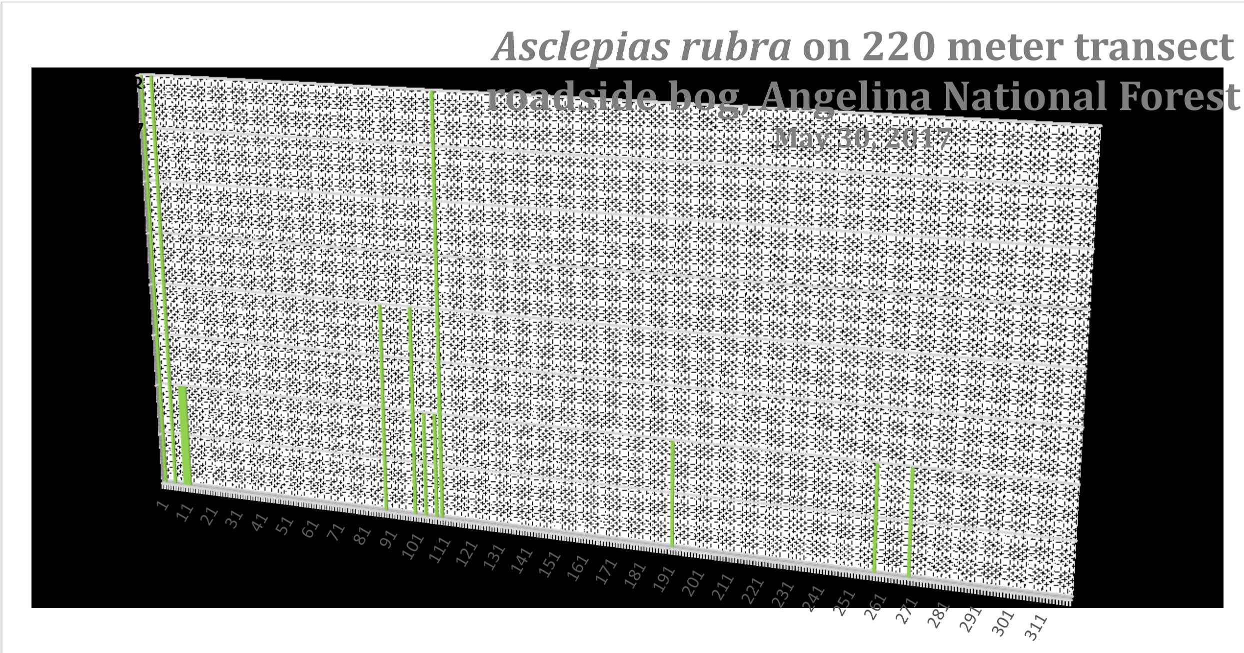


Figure6X: The only abundant population of this species located thus far has clumpy distribution in a roadside bog.

8. Value added: *Survey for "fifth generation" monarch butterflies*

While it is impossible to thoroughly survey a region as large as East Texas, we found evidence in both years of our study that this region has "fifth generation" butterflies, and it is likely that this is not rare. Besides finding fifth generation butterflies, there appears to be almost continuous generations at sites of tropical milkweeds as is discussed below.

Through our surveys and the surveys of our network of citizen scientists, we have records of 3,572 monarch butterfly sightings. These data are all uploaded onto the iNaturalist website (https://www.inaturalist.org) and are freely available for any researcher and the general public. In our effort to understand milkweed distribution that corresponds with potential reproduction sites, there are also records of all milkweed species in Texas. The milkweed data is being accumulated and georeferenced for a later distribution map of the *Asclepias* of Texas, much of which is included above in this report. In both years we observed small numbers of carterpillars in the fall at native milkweed plants, suggesting that there is continued Fall reproduction in East Texas, although this is reproduction is not nearly as extensive as in the Spring.

Signature:

Title:	Professor
Date:	30 August 2018