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Development of a DNA Barcoding Reference Library for Identification of Medicinal Plant Materials Used in the Rio Grande Valley of Texas: a Representative Case Study Using Arnica (Asteraceae)

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DEVELOPMENT OF A DNA BARCODING REFERENCE LIBRARY FOR IDENTIFICATION OF MEDICINAL PLANT MATERIALS USED IN THE RÍO GRANDE VALLEY OF TEXAS: A REPRESENTATIVE CASE STUDY USING *ARNICA (ASTERACEAE)*

BY

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Abstract

DNA barcoding is a technique that uses a short DNA fragment to identify a specimen to the species level. This technique is essential in situations where a lack of distinguishing morphological characteristics makes identification impossible. In the Río Grande Valley a variety of herbal supplements are cheap, readily available and sold as "Arnica" with no information to identify the contents. The appearance of dried and shredded material suggests that a variety of plant species are involved, belonging to the family Asteraceae. *Arnica montana*, also part of Asteraceae, is found in Europe and has anti-inflammatory properties used to externally treat bruises and contusions. Many species in Asteraceae contain secondary metabolites that may be hepatotoxic. From a health perspective, it is important that these products are identified to rule out safety concerns of toxicity of potentially mixed-up or misidentified materials. In this study a DNA barcoding reference library of Río Grande Valley Asteraceae was developed and subsequently a Bayesian phylogenetic approach was used to identify these unknown plant samples. The approach consisted of using matK and rbcL sequence data to identify the samples. The Bayesian phylogenetic tree confirmed the samples were not *A. montana*, but instead identified one species to be *Trixis inula*, and the remaining species were narrowed down to the subtribal level. Having obtained this information, additional analyses were conducted with highly variable nuclear ribosomal spacer sequences within those subtribes to further narrow down the possibilities. As a result the other samples were identified as *Heterotheca subaxillaris*, *Grindelia spp.* and *Pseudogynoxys spp.* A literature search revealed that species within each of the genera identified possess antioxidant, anti-inflammatory, anti-bacterial, anti-fungal and anti-parasitic properties some of which are highly similar to those of *A. montana.* The evidence obtained in this study suggests that these "Arnica" plants are not random replacements or misidentifications, as has been found in similar studies in other parts of the country, but are so far unrecognized members of medicinal plants widely used in the Río Grande Valley. This finding is warranting a much more detailed and molecular data driven ethnobotanical study of medicinal plant use in the RGV.

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CHAPTER I

Introduction

The use of medicinal plants to treat common maladies has occurred throughout human history. In the Río Grande Valley of Texas this is no exception. With rising healthcare costs and high poverty levels in this area, local herbal products offer an economical alternative in the treatment of common ailments. *Arnica* is an example of the many herbal products that are readily available at extremely affordable prices. In local yerberías and supermarkets packets with shredded and dried plant materials are sold as "Arnica" with no reference to any scientific name. While morphological characteristics differ among samples, suggesting different species identities, they appear to be members of Asteraceae. Their shredded nature, however, makes traditional methods of identification impractical.

Arnica is among the 1600-1700 genera of Asteraceae (Compositae), one of the largest plant families with approximately 24,000 species. A collection of florets that constitute a capitula surrounded by bracts characterizes this family (Funk et al. 2009). Species are of economic importance because of their commercial, horticultural, and medicinal uses. In South Texas, specifically, the Río Grande Valley, approximately 150 taxa of Asteraceae are found in Cameron, Hidalgo, Starr, and Willacy counties (Richardson and King 2011, Richardson unpublished data). These species embody 5 subfamilies, 18 tribes, and 100 genera giving a good representation in diversity of this large plant family.

Medicinal *Arnica* (*Arnica montana*), that has been used for centuries, is an herb native to European alpine meadows whose flowers and leaves are commonly employed in the preparation of ointments and poultices for external treatment of inflammation, bruises, and sprains (Ganzera et al. 2008, Pljevljakušić et al. 2013). Those *Arnica* preparations or plant products, however, come mostly from wild collected sources. Specialized permits are required for collection due to the endangered status of the species in several European countries (Lange 1998, Kathe 2006) and plants that are sustainably collected in Europe or come from the few

developed plantings fetch a high price (Kathe 2006). In Germany, one kilogram of flowers costs the equivalent of USD $56 in retail trade (Lange 1998). The high price for collection and import to the United States and non-availability of sufficient material for a large market often results in the collection of other plant species that are used to replace the product. Trying to find plant products with similar medicinal properties can be a legitimate endeavor, and in some cases it is successful. However, in other instances, the inability to find a legitimate replacement can lead to the intentional replacement with useless or even toxic adulterants that have similar appearance or smell simply for profit reasons. Authentication of herbal products, especially processed samples, is therefore important.

Legitimate replacements of medicinal plants have been observed throughout history. One example is the development of aspirin from knowledge that species in the genus *Salix (Salicaceae)* contain the active ingredient salicylic acid (Mahdi 2010). While initial production of this medicine was expensive, by the end of the 1890s Felix Hoffmann from the Bayer Company had found an inexpensive way to synthesize acetylsalicylic acid (DeKornfeld, 1964). The same, similar compounds or other chemical constituents with similar function cans are found in *Myrtus communis* (Myrtaceae; Mackowiak 2000), and *Filipendula ulmaria* (Rosaceae) which was formerly classified in the *Spirea* genus from which aspirin may have gotten its name (Mueller and Scheidt 1994). Another instance of a successful substitution of medicinal plants can be found in Asteraceae. For example, *Echinacea angustifolia* (Asteraceae), which has antibacterial properties and is mostly wild collected, can successfully be replaced with *Echinacea purpurea* (Asteraceae) a species that is more likely to be cultivated and have the same medicinal effects (Taylor 1996). Likewise, caffeine is found in the genus *Coffea* (Rubicaceae), *Cola acuminata* (kola nut, Malvaceae), *Ilex paraguariensis* (yerba mate, Aquifoliaceae), and *Paullinia cupana* (guaraná, Sapindaceae). A similar compound, theobromine, with slightly different properties is found in *Theobroma cacao* (cacao plant, Malvaceae). Different species of *Ilex* have been traditionally used in different geographical areas for the same purpose due to their caffeine content. *Ilex paraguariensis* (yerba mate) is widely used

in South America, *Ilex guayusa* is used in Equador for a tea, *Ilex vomitoria* (yaupon) was used by Southeastern Native Americans as a ceremonial stimulant, and *Ilex kundingcha* is used to prepare a tea in China.

The above examples illustrate that substitutions for commonly used medicinal plants can work as long as they do not contain additional chemical compounds that are toxic or show other unwanted properties. Those potential toxic compounds, unfortunately, can be common in Asteraceae. Sesquiterpene lactones (SLs) and pyrrolizidine alkaloids (PAs) are known secondary metabolites of Asteraceae (Calabria et al 2009) often found in that family. In studies SLs have demonstrated anti-parasitic, anti-feedant, anti-bacterial, anti-fungal, and anti-tumor properties (Picman 1986). One of the active components in *Arnica montana* is the sesquiterpene lactone helenalin (Lyß et al. 1998, Pljevljakušić et al. 2013). Artemisinin, also a SL, is obtained from *Artemisia annua* and used medicinally to treat malaria (Teoh et al. 2006). SLs, however, may exhibit adverse effects such as contact dermatitis and toxic effects at low concentrations. A study conducted on patients who exhibited contact dermatitis when exposed to SLs showed many also developed allergic reactions to the constituents of teas derived from plants in Asteraceae (Lundh et al. 2006). Cases of accidental and deliberate ingestion of plants containing SLs by humans and livestock leading to poisoning or death have been reported. Species found in the Río Grande Valley such as *Helenium microcephalum*, *Parthenium hysterophorous* and *Hymenoxys* species are culprits in cases of livestock poisonings (Picman 1986). Cases of human poisonings attributed to contamination of bread with seeds from *Helenium amarum* have also been reported (Kingsbury 1964, Picman 1986).

Pyrrolizidine alkaloids (PAs) are also present in many species of Asteraceae, specifically in the tribes Senecionae and Eupatorieae (Calabria et al. 2009). Representatives of both tribes are found in the Río Grande Valley, such as *Senecio riddellii*. Principally, PAs affect the liver and may subsequently affect the lungs as pyrroles formed by the metabolism in the liver can make their way to the lungs (Prakash et al. 1999). They have been associated with hepatic veno-occlusive disease, hepato-splenomegaly, emaciation in chronic cases and possible hepatotoxicity in

fetuses (Prakash et al. 1999). Like SLs, PAs also harm and even kill livestock especially pigs and poultry and in laboratory studies have been linked to cancer in rats (Prakash et al. 1999). Given the potential health issues associated with those secondary metabolites, correct plant identification and non-toxic substitutions are of utmost consumer importance.

A relatively new method of species identification is DNA barcoding, which uses a combination of short and highly variable standardized sequence fragments of an organism's genome (Hebert et al. 2003). The technique seeks to be a quick and reliable form of identification that would simplify cumbersome and time-consuming traditional methods and bring its applications to the hands of a wider public including non-experts. DNA barcoding has been applied across various fields to address authentication of products, to study biodiversity, and to address issues of invasive and endangered species among many others. The authentication of products has included kitchen spices in Lamiaceae (De Mattia et al. 2010), and identification of ingredients of commercial teas (Stoeckle et al. 2011). Biodiversity studies have employed DNA barcoding to identify plant species in a forested area in Panama (Kress et al. 2009) and to discriminate plant species in a temperate flora (Burgess et al. 2011). As a means for biosecurity, authorities in New Zealand used DNA barcodes to identify potential pest species that can damage crops and result in a heavy economic burden (Armstrong and Ball 2005). In cases of endangered species barcodes have been used to distinguish species of ginsengs (Zuo et al. 2011) and cycads (Sass et al. 2007). This can help prevent their illegal trade given many species are listed on the Convention on International Trade in Endangered Species (CITES) list. As more species are barcoded, a larger database can be compiled thus increasing the potential of the movement.

The use of DNA barcodes in animals, which are based on the sequence diversity in the mitochondrial gene cytochrome c oxidase subunit 1 (COI) has advanced faster than in plants, discriminating closely related species most animal phyla with some exceptions such as Cnidaria (Hebert et al. 2003). In plants, unfortunately, this approach has not been viable because plant mitochondrial genes

exhibit low rates of sequence change and do not show good species-level resolution (Kress 2005, Chase 2005, Newmaster et al. 2006, Fazekas et al. 2008). Researchers have therefore turned to several chloroplast markers (Kress and Erickson 2008). Because no single marker has had the same success as COI, researchers have supported the use of multiple genome regions (Chase et al 2005, Kress et al. 2005, Cowan et al. 2006, Newmaster et al 2006, Kress and Erickson 2007).

Barcoding regions in the chloroplast that are utilized include the non-coding intergenic plastid spacers (atpF-atpH, psbK-psbI, trnH-psbA), and coding regions (accD, matK, ndhJ, rbcL, rpoB, rpoC1 and ycf5). The nuclear ribosomal internal transcribed spacer (ITS 1, 5.8S, and ITS 2) has also been employed in some studies. While there seems to be more variation in non-coding regions compared to coding regions, alignment issues have surfaced with the non-coding regions. In 2009 the Consortium for the Barcoding of Life recommended the use of maturaseK (matK) and ribulose-bisphosphate carboxylase (rbcL) as standard markers in plants (CBOL Plant Working Group, 2009). The gene rbcL encodes for the large subunit of ribulose-1-5-bisphosphate carboxylase, a key enzyme in photosynthesis (Suzuki and Makino, 2013). The gene matK is nested within the trnK intron, which serves as a maturase of type II introns from RNA (Hausner et al. 2006). The use of matK has had high success rates in diverse plant groups such as a study on *Tolpis* (Asteraceae) by Mort et al. (2010), (Zingiberaceae) by Selvaraj et al. (2008) and various poisonous plant species by Bruni et al. (2010)

Authentication (Figure1) can start after sequence information from unknown samples is obtained. BLAST (Basic Local Alignment Search Tool) (Altschul et al. 1990) and phylogenetic analysis are two ways to authenticate plant material. A BLAST search can yield a perfect match or a close match. A perfect match can mean a sample has been identified, on the contrary it may mean that the sequences are identical but belong to different species. In a close match, non-identical sequences may belong to the same species due to intraspecific variation or belong to different species. Phylogenetic analysis can in turn yield two outcomes. An unknown sample may group with a monophyletic group of conspecifics and be considered identified.

On the other hand it may group with a clade of its closest relatives, all of different species. When this occurs, a subtree containing all the genera and species represented must be generated for that particular region of the tree. The statistical support and fact that this method does not base itself on similarity like BLAST make it a better method.

Asteraceae is a suitable candidate for a proof of principle test because it is a taxonomically challenging family to work with due to the copious number of species and general low levels of genetic and morphological variability that distinguish the different taxa. Given the inexpensive nature and wide availability of "Arnica" herbal supplements in the Río Grande Valley, it is important that consumers know the identity of these products, especially if ingesting them can be harmful.

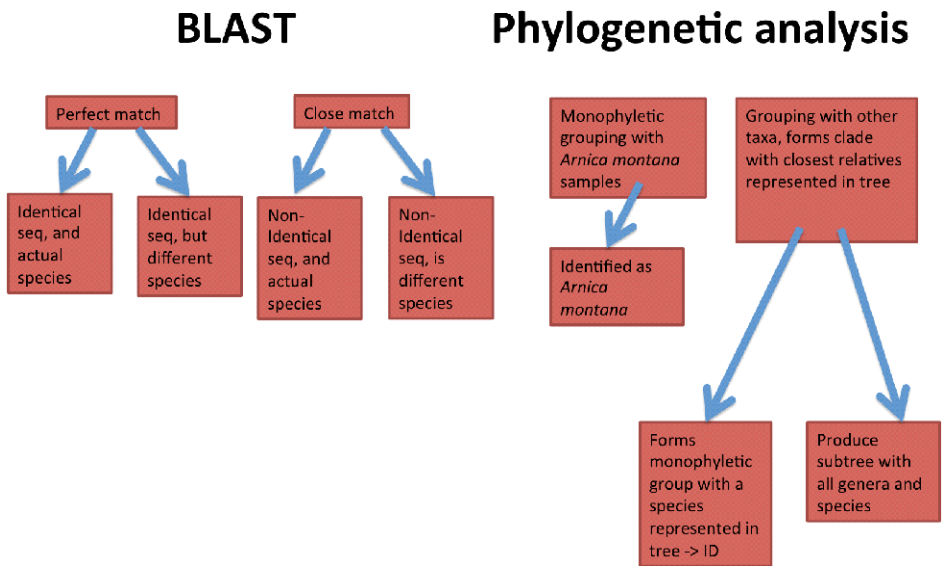

Figure 1 The process of authentication of an unidentified plant sample. The diagram indicates the differences in steps taken when using the Basic Local Alignment Search Tool (BLAST) versus phylogenetic tree analysis to identify an unknown sample.

Study Purpose

This study aims to 1) build a DNA Barcode reference library for the Asteraceae of the Río Grande Valley; 2) identify the samples sold under the name "Arnica" through phylogenetic analyses of DNA Barcodes for matK and rbcL; 3) determine if the possibility of these plants being locally collected exists; 4) compare the results given from a phylogenetic tree to those of a nucleotide BLAST search.

Hypotheses

1) "Arnica" samples sold for medicinal purposes in the Río Grande Valley are not *Arnica montana*

2) "Arnica" herbal supplements may be species of locally collected Asteraceae that replaced *Arnica montana*.

3) A Bayesian phylogenetic approach is superior to a BLAST search for correctly authenticating plant material.

CHAPTER II

Materials and Methods

Specimen collection

Species in the Asteraceae of the Río Grande Valley were collected in accordance with the checklist of all Asteraceae provided by Dr. Alfred Richardson. The majority of the specimens were collected from 2010 to 2012 in the four counties that make up the Río Grande Valley: Cameron, Hidalgo, Starr and Willacy. The samples were identified, pressed and mounted on special archival paper as voucher specimens to be stored in the Runyon Herbarium of the University of Texas at Brownsville. Samples from the Runyon Herbarium and the Herbarium at the University of Texas Pan American were used to represent the species not found in the field. Leaves from the specimens were placed in envelopes labeled with the corresponding species name, date collected and location.

DNA isolation

Dried leaves from field collected plants and herbarium specimens were gathered and weighed to obtain approximately 25 mg per specimen for each extraction. The samples were placed in 2 ml Eppendorf centrifuge tubes with a 3mm Qiagen tungsten carbide bead and stored in an ultracold freezer for a minimum of 48 hours at -80°C. The frozen plant tissue was disrupted with a Qiagen TissueLyser (Qiagen, Valencia, CA) for 2 intervals of 1 minute at 25 Hz.

For field collected specimens and those from the Runyon Herbarium, whole genomic DNA was extracted with a Qiacube robot (Qiagen, Valencia, CA) using the Qiagen DNeasy Plant Mini Kit following the manufacturer's instructions. The DNA was stored in Tris-EDTA buffer at pH8 and kept in a freezer for further processing. Whole genomic DNA for samples from the Herbarium of the University of Texas Pan American and samples from the Runyon herbarium that failed to amplify were

extracted with the NucleoSpin 96 Plant II kit (Machery-Nagel, Duren, Germany) following the manufacturer's instructions). Upon the extraction of DNA, the samples' absorbance ratio and concentrations were measured and recorded with a Thermo Scientific NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc Wilmington, Delaware, United States). A working stock was diluted in water to a concentration of 20ng/µl with ddH2O unless the concentration of DNA was lower than 5 ng/ µl , at which point the DNA was used directly from the stock.

DNA amplification

Polymerase Chain Reactions were conducted in a BioRad C1000 Thermal Cycler or a BioRad DNA Engine Peltier Thermal Cycler where three programs were used for reactions of 15 µl, 25ul and 50 µl for matK and rbcL. The default program and the one mostly used had an initial denaturation at 94 °C for 60 sec, 37 cycles of melting at 94 ° C for 10 sec and annealing at 50 °C for 30 sec, an extension at 72 °C for 2.45 min, followed by a final extension at 72 °C for 3 min and a final hold at 4 °C.

An second program was used when a very light band was obtained with the default program. The program consisted of an initial denaturation at 94 °C for 60 sec, 40 cycles of melting at 94 ° C for 10 sec and annealing at 50 °C for 30 sec, an extension at 72 °C for 2.45 min, followed by a final extension at 72 °C for 3 min and a final hold at 4 °C.

On rare occasions, a third program was used, it consisted of an initial denaturation at 94 °C for 60 sec, 42 cycles of melting at 94 ° C for 10 sec and annealing at 50 °C for 30 sec, an extension at 72 °C for 2.45 min, followed by a final extension at 72 °C for 3 min and a final hold at 4 °C.

Individual amplifications for field-collected specimens were done in 50 µl reactions containing: 28.7 µl of sterile ddH2O, 11.9 µl of 4.2X Tricine PCR mix (300 mM tricine, 500 mM KCl, 20 mM MgCl2, pH to 8.4 with 10 M KOH, 0.5 µl (100 µM) of forward primer, 0.5 µl (100 µM) of reverse primer, 0.4 µl of Taq polymerase (2.5 µ/µl), and 8µl of genomic DNA (20ng/µl).

Amplifications for species obtained from the Runyon Herbarium were done in

25 µl. If the species amplified, the remainder of the product was obtained in several additional 50 µl reactions. The PCR mix for 25 µl reactions consisted of: 17.3 µl of sterile ddH2O, 6.0 µl of 4.2X Tricine PCR mix [dNTPs], 0.25 µl (100 uM) of primer 1, 0.25 µl (100 µM) of primer 2, 0.20 µl of Taq polymerase (2.5 µ/µl), and 4.0 µl of genomic DNA (20ng/µl). For species that did not amplify even after different primer combinations, 5 µl of 5X TBE-PAR additive (Samarakoon et al. 2012) were used with the above protocol but subtracting the same amount from the ddH2O.

Because of the limited nature of plant tissue obtained from the Herbarium at the University of Texas Pan American, initial amplifications were done in 15 µl. In cases where the species amplified, more product was made in 25 µl reactions. When a species had amplified in 15 µl reactions and subsequently did not amplify in 25 reactions, the entire product was obtained from 15 µl reactions. The mix for 15 µl reactions consisted of: 8.6 µl of sterile ddH2O, 3.6 µl of 4.2X Tricine PCR mix [dNTPs], 0.15 µl (100 µM) of forward primer, 0.15 µl (100 µM) of reverse primer, 0.12 µl of Taq polymerase (2.5 µ/µl), and 2.4 µl of genomic DNA (20ng/µl). For species that did not amplify even after different primer combinations, 3 µl of 5X PAR additive were used with the above protocol but subtracting the same amount from the ddH2O.

Primers

Initial PCRs were conducted using matK primers 2F, 1R, as recommended from Phase 2 of Kew Barcoding protocols (http://www.kew.org/barcoding/protocols.html). Default rbcL primers were rbcL 1F (Olmstead et al. 1992) and rbcL 1460R. Several primers were designed using Primer3Plus software program in order to use with species that did not amplify with the original set of primers. A list of primers can be seen in Table 1. Their visual representations are shown in Figures 2 and 3.

Table 1 **Sequences of primers used in the study**

Primer Name	**Primer Sequence**
Original Primers:	
rbcL 1F	5'- ATG-TCA-CCA-CAA-ACA-GAA-ACT-AAA-GC-3'
rbcL 1460R	5'- CTT-TTA-GTA-AAA-GAT-TGG-GCC-GAG -3'
matKF2_BC	5'- ATC-CAT-CTG-GAA-ATC-TTA-GTT-C -3'
matKR1_BC	5'- GTT-CTA-GCA-CAA-GAA-AGT-CG -3'
Designed Primers:	
Comp rbcL 1R	5'- CCA-TAC-TTC-ACA-AGC-AGC-AGC-TAG-T -3'
Comp rbcL 1F	5'- CCA-AGG-ATA-CTG-ATA-TCT-TGG-CAG-C -3'
Comp rbcL 2F	5'- CCG-CTA-AAA-ACT-ACG-GTA-GAG-C -3'
Comp rbcL 2R	5'- TAC-CCG-CAG-TAG-CAT-TCA-AGT-AAT-GC -3'
Comp rbcL 1320R	5'- CAT-TTG-GTA-GCC-TCA-CGG-ATA -3'
Comp rbcL 1280R	5'-CAA-GAT-CGC-GTC-CCT-CAT-TA -3'
Comp rbcL 1198R	5'-AGG-GTG-CCC-TAA-AGT-TCC-TC -3'
Comp rbcL 1165R	5'-TAC-GGA-ATC-ATC-CCC-AAA-GA -3'
Comp rbcL 960R	5'-ACG-GTA-CCG-GAA-TGA-ATG-TG -3'
Comp rbcL 376F	5'-TGC-GAA-TCC-CTA-CTG-CGT-AT -3'
Comp rbcL 225F	5'- GAG-CCT-GTT-CCT-GGA-GAA-GA -3'
Comp rbcL 170F	5'- ATG-GAC-CGA-TGG-ACT-TAC-GA -3'
Comp matK F2a	5'- AAG-ATT-CTT-TCT-CCA-TSA-GTG-TC -3'
Comp matK F2b	5'- CTT-ACG-ATC-AAC-ATC-YTC-TGG-AGC-YC -3'
Comp matK R1a	5'- CCT-TMC-CDA-TAG-GAY-GCC-C -3'
Comp matK R1b	5'- CAC-TTR-AAY-GAT-AAC-CCA-GAA-AG -3'
matK_F2c	5'- GTT-CAA-GCT-CTT-GCC-TAT-TGG -3'
matK_R1c	5'- TCT-GAT-AAA-TCG-GCC-CAA-AC -3'
matK_F2d	5'- TCA-AGC-TCT-TCG-CTA-TTG-GA -3'
matK_R1d	5'- ATC-TGA-TAA-ATC-GGC-CCA-AA -3'

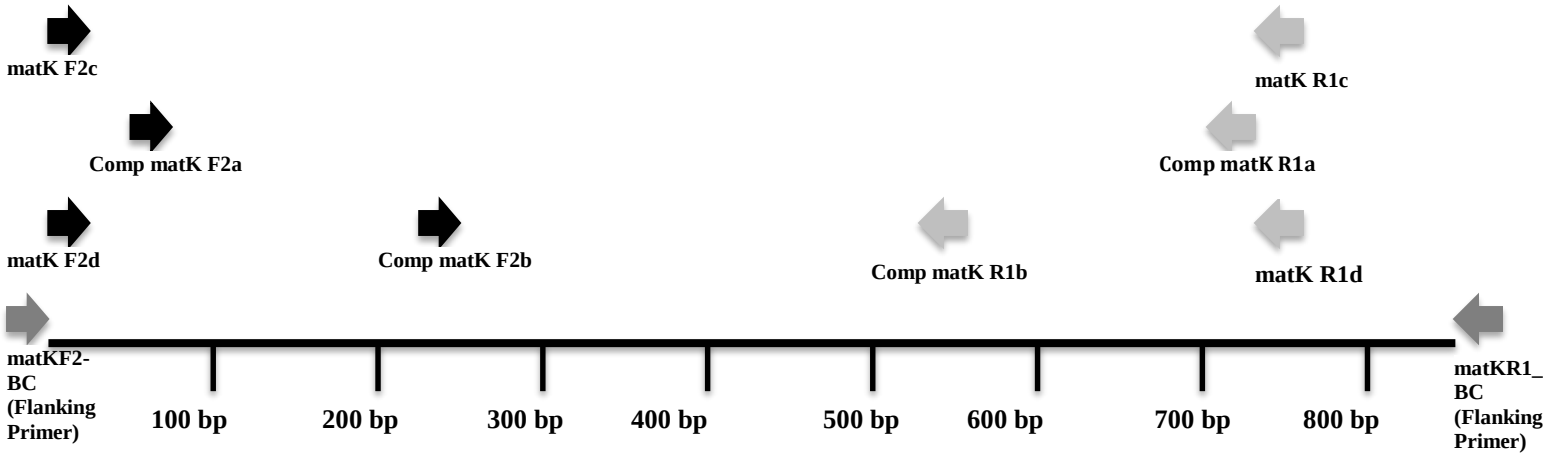

Figure 2
Primers for amplification of matK chloroplast barcode. The following map indicates the approximate position of each primer used for the matK region. Dark gray arrows indicate flanking primers. Black arrows indicate forward primers. Light gray arrows indicate reverse primers.

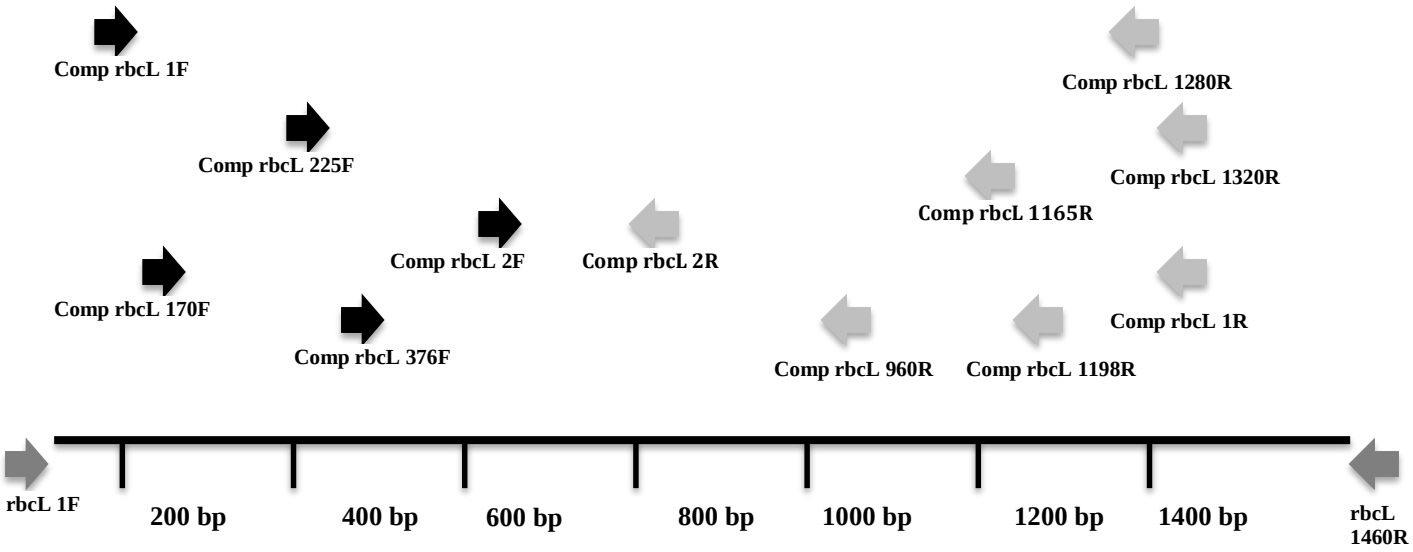

Figure 3
Primers for amplification of rbcL chloroplast gene. The following map indicates the approximate position of each primer used for the rbcL barcoding region. Dark arrows indicate flanking primers. Black arrows indicate forward primers. Light gray arrows indicate reverse primers.

PCR product clean up

Upon obtaining 150-200 µL of PCR product per sample, a Thermo Scientific Savant DNA 120 SpeedVac Concentrator set to medium drying rate was used to concentrate the samples to 30-40 µl. A volume of 10 µl of 1X loading buffer was added to each sample and they were loaded into a 1.5 percent agarose cleaning gel leaving an empty well between each sample. The gel was run at 120 V on 1X TBE buffer for 45 minutes. The second half of the samples were then loaded in the empty wells and the gel was run for another 45 minutes. The separation of the samples was done to simplify the cutting of the bands. After 90 minutes the gel was removed and placed on a Fisher Scientific UV transilluminator where the DNA bands were cut out one at a time and placed in a 2 ml Eppendorf centrifuge tube. PCR products that were not being cut were protected with aluminum foil and the light was turned off between cuttings. The samples were then cleaned using a QiaQuick Gel Extraction kit with a Qiacube robot according to protocol.

Sequencing

Samples were loaded in a sequencing plate with the same forward and reverse primers used for PCR diluted at 100mM/µL. The samples were sent to McLab Sequencing Facility in South San Francisco, California for sequencing with an automated capillary DNA sequencer. The length of the partially sequenced matK barcode ranged from 479-811 bp and 452-1429 bp for rbcL. The length of the sequences was dependent on sequencing success. The published sequence lengths were 832 bp for matK and 1460 bp for rbcL.

Sequence editing

Raw sequences from McLab were manually edited, assembled into contigs and manually aligned using Sequencher 4.10.1 (Gene Codes Corporation).

Data sources

Data sources for this study were obtained from a variety of sources, some

were provided by Dr. Andrea Schwarzbach, (UT Brownsville unpublished data), others were sequenced, and additional sequences were obtained from GenBank (Benson et al. 2007).

ITS, matK and rbcL sequences for unidentified "Arnica" samples, and control sequences of *A. montana* (Arnica samples 20055, Arnica 20056) were provided by Dr. Andrea Schwarzbach, (UT Brownsville unpublished data) (Table 2). The data that were sequenced for this study and used in the Bayesian phylogenetic analysis of the family can be found in Table 3 A along with each species, accession number and the length of matK and rbcL sequence. Additional sequences that served as outgroups, and sequences to supplement the ones sequenced for the study were obtained from GenBank (Table 3 B). In cases where only one marker amplified, a representative of the same species was obtained from GenBank and paired with the species amplified in this study (Table 3 C). The unidentified "Arnica" samples provided by Dr. Andrea Schwarzbach as well as sequences obtained from GenBank were used to create an ITS subtrees. The subtrees were used to identify Arnica sample 20077 within subtribe Senecioninae (Table 4), Arnica sample 20029 within Chrysopsidinae (Table 5), and Arnica sample 20057 within Machaerantherinae Table 6).

Table 2 Unknown "Arnica" samples and controls for Bayesian Family Analysis

Species	Accession Number	matK length (bp)	rbcL length (bp)	ITS length (bp)
Pseudogynoxys chenopodioides	1989	803	MISSING	MISSING
Heterotheca subaxillaris	Arnica 20029	810	1415	638
Arnica montana	Arnica 20055	806	1413	N/A
Arnica montana	Arnica 20056	771	1416	N/A
Grindelia ssp.	Arnica 20057	804	1414	644
Trixis ssp.	Arnica 20063	798	1412	N/A
Pseudogynoxys ssp.	Arnica 20077	803	1412	579

Table 3A List of sequences obtained for Bayesian family analysis

Species	Accession Number	matK length (bp)	rbcL length (bp)
Acourtia runcinata	40	687	710
Acourtia wrightii	125	724	1294
Amblyolepis setigera	65	707	851
Ambrosia confertifolia	67	674	1201
Ambrosia trifida	174	707	1203
Aphanostephus skirrhobasis	26	802	1210
Artemisia ludoviciana	175	707	1287
Arnica amplexicaulis	179	721	1332
Baccharis halimifolia	69	803	1273
Baccharis halimifolia	132	793	1370
Baccharis salicifolia	70	810	1120
Baccharis texana	71	809	1257
Bahia absinthifolia	42	771	1387
Bidens laevis	181	722	1003
Borricchia frutescens	29	771	1387
Calyptocarpus vialis	28	699	1206
Chaetopappa asteroids	58	809	1375
Chaetopappa bellidifolia	182	762	1330
Chromolaena odorata	52	771	1209
Chloracantha spinosa	136	700	1375
Circium texanum	44	793	1353
Clappia suaedifolia	74	732	1282
Clappia suaedifolia	122	773	1383
Conoclinum betonicifolium	128	756	1379
Conyza canadensis	75	737	1123
Conyza canadensis	127	799	1375
Coreopsis tinctoria	5	807	1360
Coreopsis tinctoria	16	694	1206
Croptilon rigidifolium	23	770	1209
Diaperia verna	22	807	1414
Emilia fosbergii	109	803	1383
Engelmannia peristenia	126	684	1288
Erigeron procumbens	21	803	1388
Erigeron tenellus	78	803	738
Eupatorium compositifolium	185	763	1333
Euthamia gymnospermoides	184	733	1090
Flaveria brownii	79	803	1417
Flaveria brownii	186	746	1086
Fleishmannia incarnata	55	771	1213
Florestina tripteris	36	722	1251
Gaillardia pulchella	17	803	1375
Gaillardia suavis	81	722	1353
Gamochaeta calviceps	62	806	1199
Gamochaeta pensylvanica	187	738	1099
Gutierrezia sphaerocephala	85	809	1198
Gymnosperma gymnosperma	87	803	1284
Helenium amarum	88	798	1409
Helenium amarum	188	524	1006
Helenium microcephalum	57	771	1196
Helianthus argophyllus	124	734	1416

Table 3A Continued List of sequences obtained for Bayesian family analysis

Species	Accession Number	matK length (bp)	rbcL length (bp)
Helianthus praecox	35	732	1210
Heterotheca subaxillaris	24	801	1426
Heterotheca subaxillaris	49	771	1198
Hymenopappus artemisiifolius	59	649	1219
Hymenoxys odorata	41	771	1387
Isocoma coronopifolia	91	750	1282
Isocoma coronopifolia	140	810	1383
Isocoma drummondi	141	786	1383
Isocarpha oppositifolia	90	729	1284
Iva angustifolia	134	740	1211
Iva annua	135	773	1204
Jefea brevifolia	177	728	1208
Laenecia coulteri	173	708	1090
Launeae intybacea	94	787	512
Launaea intybacea	133	742	1375
Liatris elegans	155	707	1206
Melampodium cinereum	120	726	1411
Melampodium cinereum	157	651	1079
Packera tampicana	10	792	1387
Palafoxia hookeriana	158	708	1124
Palafoxia texana	34	807	1384
Parthenium incanum	96	707	1250
Pectis angutifolia	39	803	1414
Perityle microglossa	19	557	710
Plectocephalus americanus	72	653	1002
Plectocephalus americanus	178	729	1065
Pluchea carolinensis	47	737	1347
Pluchea odorata	112	728	1202
Pseudognaphalium austrotexanum	97	803	1212
Pseudognaphalium stramineum	46	771	1383
Pyrrhopappus pauciflorus	15	799	1413
Ratibida columnifera	2	779	1414
Ratibida columnifera	37	807	1414
Ratibida peduncularis	53	771	1389
Rayjacksonia phyllocephala	56	786	1383
Rudbeckia hirta	61	771	1377
Sanvitalia ocymoides	138	695	1383
Sclerocarpus uniserialis	32	775	1375
Senecio ampullaceus	12	734	1298
Simsia calva	8	804	1384
Simsia calva	162	670	1128
Solidago canadensis	102	624	1325
Solidago canadensis	164	734	768
Solidago sempervirens	45	731	1380
Sonchus asper	25	791	1210
Sonchus oleraceaus	11	806	1384
Symphyotrichum subulatum	1	711	1217
Tamaulipa azurea	48	569	1383
Taraxacum officinale	103	670	1289
Tetragonotheca repanda	20	774	1386

Table 3A Continued List of sequences obtained for Bayesian family analysis

Species	Accession Number	matK length (bp)	rbcL length (bp)
Tetraneuris linearifolia	167	706	1080
Thelesperma ambiguum	168	704	1251
Thelesperma filifolium	170	707	1080
Thelesperma megapotamicum	169	479	452
Thymophylla tephroleuca	107	699	1209
Thymophylla tephroleuca	64	803	1412
Trichocoronis wrightii	105	706	729
Trichocoronis wrightii	171	706	1124
Tridax procumbens	111	711	1426
Trixis inula	106	788	1298
Trixis inula	130	799	1287
Varilla texana	43	779	715
Verbesina encelioides	33	808	1387
Verbesina microptera	131	802	1344
Viguiera stenoloba	7	771	1416
Wedelia acapulcensis	9	786	1206
Xanthisma texanum	60	809	1406
Xanthium strumarium	172	707	1130
Zinnia acerosa	30	771	1388
Amphiachyris dracunculoides	176	MISSING	457
Diaperia candida	113	MISSING	1259
Heterotheca canescens	82	MISSING	1257
Neonesomia palmeri	129	MISSING	1381
Pterocaulon virgatum	159	MISSING	523
Thelesperma nuecense	27	MISSING	1414
Ambrosia psilostachya	66	698	MISSING
Coreopsis nuecensis	13	725	MISSING

Table 3B Sequences from GenBank for Bayesian Analysis

Species	GenBank Number	matK length (bp)	GenBank Number	rbcL length (bp)
Adenophora divaricata (Outgroup)	EU713323.1	810	EU713430.1	1362
Adenophora liliifolioides(Outgroup)	JN 851163.1	546	JN851172.1	1308
Acicarpha spathulata (Outgroup)	EU385316.1	810	EU384939.1	1425
Boopis anthemoides (Outgroup)	EU841363.1	810	EU384978.1	1376
Moschopsis rosulata (Outgroup)	GQ983662.1	790	X87390.1	1179
Arnica mollis	AY215764.1	811	AY215084.1	1380
Arnica dealbata	AY215880.1	811	AY215753.1	1380
Arnica angustifolia	JN966119.1	768	KC482034.1	552
Achillea millefolium	EU385315.1	804	JX848399.1	1382
Ambrosia artemisiifolia	HQ593164.1	811	DQ006055.1	683
Anthemis cotula	HM850611.1	772	HM849779.1	1367
Baccharis neglecta	EU385326.1	811	EU384949.1	1425
Emilia sonchifolia	HM989795.1	797	JQ933323.1	1387
Gochnatia hypoleuca	EU385357.1	811	EU384978.1	1425
Solidago gigantea	HQ593451.1	775	HM850369.1	1367
Soliva anthemifolia	HM989766.1	797	JQ933485.1	1385
Trixis divaricata	EU385405.1	811	EU385025.1	1410
Trixis inula	JQ586936.1	756	JQ590725.1	552

Table 3C Sequences from GenBank and collected pair for Bayesian Analysis

Species	GenBank or Accession Number	matK length (bp)	GenBank or Accession Number	rbcL length (bp)
Pectis cylindrical	KJ525232.1	811	MISSING	N/A
Egletes viscosa	JQ586845.1	761	MISSING	N/A
Flaveria trinervia	MISSING	N/A	HQ534146.1	1463
Xanthisma spinulosum	MISSING	N/A	JX848434.1	1382
Grindelia squarrosa	MISSING	N/A	JX848414.1	1373
Youngia japonica	Obtained for study (108)	799	EU385029.1	1376
Gamochaeta pensylvanica	Obtained for study (18)	759	EU384977.1	1429
Bidens pilosa	Obtained for study (4)	632	HM849815.1	704
Eclipta prostrata	Obtained for study (63)	811	AY215108.1	1386
Helianthus annuus	Obtained for study (6)	771	LI3929.1	1425
Parthenium hysterophorus	Obtained for study (51)	723	JQ933433.1	1387
Psilostrophe gnaphalodes	Obtained for study (98)	700	AY215168.1	1380
Thymophylla tenuiloba	AY215787.1	811	Obtained for study (14)	1406
Coreopsis basalis	AY551492.1	811	MISSING	N/A

Table 4 List of GenBank Sequences for Senecioninae Subtree

Senecioninae	GenBank Accession Number
Pseudogynoxys benthamii	AF459958.1
Charadranaetes durandii	EF538164.1
Dorobaea laciniata	EF538187.1
Garcibarrigoa telembina	EF538211.1
Jessea multivenia	EF538246.1
Jessea cooperi	EF538245.1
Misbrookea strigosissima	EF538254.1
Pseudogynoxys haenkei	EF538288.1
Pseudogynoxys chenopodioides	EF538287.1
Senecio arnaldii	EF538297.1
Werneria nubigena	EF538413.1
Werneria caespitosa	EF538412.1
Xenophyllum poposum	EF538415.1
Xenophyllum dactylophyllum	EF538414.1
Talamancalia boquetensis	EF538403.1

Table 5 List of GenBank Sequences for Chrysopsidinae Subtree

Senecioninae	**GenBank Accession Number**
Croptilon divaricatum	AF251576.1
Chrysopsis gossypina	AF046993.1
Heterotheca villosa	AF046994.1
Geissolepis suaedifolia	DQ478972.1
Noticastrum marginatum	DQ478975.1
Heterotheca cf. subaxillaris	EF190033.1
Chrysopsis mariana	GQ892729.1
Heterotheca subaxillaris	GQ892730.1
Pityopsis aspera	GQ892714.1
Pityopsis aspera	GQ892713.1
Pityopsis aspera var. *adenolepis*	GQ892715.1
Pityopsis aspera var. *adenolepis*	GQ892716.1
Pityopsis falcata	GQ892702.1
Pityopsis falcata	GQ892703.1
Pityopsis falcata	GQ892704.1
Pityopsis flexuosa	GQ892710.1
Pityopsis graminifolia var. *aequifolia*	GQ892722.1
Pityopsis graminifolia var. *aequifolia*	GQ892723.1
Pityopsis graminifolia var. *aequifolia*	GQ892724.1
Pityopsis graminifolia var. *graminifolia*	GQ892718.1
Pityopsis graminifolia var. *graminifolia*	GQ892717.1
Pityopsis graminifolia var. *latifolia*	GQ892726.1
Pityopsis graminifolia var. *latifolia*	GQ892728.1
Pityopsis graminifolia var. *latifolia*	GQ892727.1
Pityopsis graminifolia var. *latifolia*	GQ892725.1
Pityopsis graminifolia var. *tenuifolia*	GQ892719.1
Pityopsis graminifolia var. *tenuifolia*	GQ892721.1
Pityopsis graminifolia var. *tenuifolia*	GQ892720.1
Pityopsis pinifolia	GQ892705.1
Pityopsis pinifolia	GQ892707.1
Pityopsis pinifolia	GQ892706.1
Pityopsis oligantha	GQ892712.1
Pityopsis oligantha	GQ892711.1
Pityopsis ruthii	GQ892708.1
Pityopsis ruthii	GQ892709.1
Heterotheca villosa	HQ142622.1
Croptilon rigidifolium	U97606.1
Heterotheca fulcrata	U97615.1

Table 6 List of GenBank Sequences for Machaerantherinae Subtree

Machaerantherinae	GenBank Accession Number
Eurybia wasatchensis	DQ478985.1
Pyrrocoma clementis	DQ478982.1
Machaeranthera tanacetifolia	DQ478981.1
Rayjacksonia phyllocephala	DQ478983.1
Isocoma tenuisecta	DQ478980.1
Grindelia nana	DQ478979.1
Machaeranthera tagetina	JQ011982.1
Isocoma menziesii	JQ011983.1
Xanthisma spinulosum var. *chihuahuanum*	JQ011999.1
Xanthisma spinulosum	JQ011984.1
Rayjacksonia phyllocephala	JQ012000.1
Hazardia squarrosa var. *grindelioides*	JQ012001.1
Pyrrocoma racemosa var. *sessiliflora*	JQ011981.1
Dieteria canescens var. *aristata*	JQ011980.1
Corethrogyne filaginifolia	JQ011998.1
Haplopappus anthylloides	JQ011975.1
Haplopappus setiger	JQ011979.1
Haplopappus undulatus	JQ011971.1
Haplopappus uncinatus	JQ011972.1
Haplopappus multifolius	JQ011973.1
Haplopappus multifolius	JQ011974.1
Haplopappus glutinosus	JQ011978.1
Haplopappus glutinosus	JQ011976.1
Haplopappus velutinus	JQ011977.1
Grindelia adenodonta	JQ011939.1
Grindelia adenodonta	JQ011985.1
Grindelia anethifolia	JQ011946.1
Grindelia anethifolia	JQ011947.1
Grindelia aphanactis	JQ011909.1
Grindelia aphanactis	JQ011927.1
Grindelia aphanactis	JQ011928.1
Grindelia arizonica	JQ011943.1
Grindelia boliviana	JQ011949.1
Grindelia brachystephana	JQ011948.1
Grindelia brachystephana	JQ011951.1
Grindelia brachystephana	JQ011954.1
Grindelia buphthalmoides	JQ011959.1
Grindelia buphthalmoides	JQ011968.1
Grindelia camporum	JQ011896.1
Grindelia camporum	JQ011930.1

Table 6 Continued: List of GenBank Sequences for Machaerantherinae Subtree

Machaerantherinae	GenBank Accession Number
Grindelia camporum	JQ011931.1
Grindelia chiloensis	JQ011953.1
Grindelia chiloensis	JQ011957.1
Grindelia chiloensis	JQ011967.1
Grindelia chiloensis	JQ011965.1
Grindelia ciliata	JQ011940.1
Grindelia ciliata	JQ011997.1
Grindelia coronensis	JQ011962.1
Grindelia coronensis	JQ012002.1
Grindelia covasii	JQ011950.1
Grindelia covasii	JQ011955.1
Grindelia decumbens	JQ011920.1
Grindelia decumbens	JQ011921.1
Grindelia fastigiata	JQ011910.1
Grindelia fastigiata	JQ011922.1
Grindelia fastigiata	JQ011945.1
Grindelia fraxinipratensis	JQ011987.1
Grindelia fraxinipratensis	JQ011988.1
Grindelia fraxinipratensis	JQ011898.1
Grindelia globularifolia	JQ011963.1
Grindelia glutinosa	JQ011966.1
Grindelia grandiflora	JQ011995.1
Grindelia greenmanii	JQ011932.1
Grindelia hallii	JQ011990.1
Grindelia havardii	JQ011895.1
Grindelia havardii	JQ011994.1
Grindelia hirsutula	JQ011899.1
Grindelia howellii	JQ011989.1
Grindelia integrifolia	JQ011907.1
Grindelia inuloides	JQ011901.1
Grindelia laciniata	JQ011911.1
Grindelia lanceolata	JQ011908.1
Grindelia lanceolata	JQ011914.1
Grindelia lanceolata	JQ011915.1
Grindelia lanceolata	JQ011991.1
Grindelia mendocina	JQ012003.1
Grindelia nana	JQ011894.1
Grindelia nana	JQ011903.1
Grindelia nana	JQ011926.1
Grindelia nana	JQ011933.1
Grindelia nana	JQ011942.1

Table 6 Continued: List of GenBank Sequences for Machaerantherinae Subtree

Machaerantherinae	GenBank Accession Number
Grindelia nuda	JQ011913.1
Grindelia nuda	JQ011996.1
Grindelia oolepis	JQ011992.1
Grindelia oolepis	JQ011993.1
Grindelia orientalis	JQ011958.1
Grindelia oxylepis	JQ011900.1
Grindelia patagonica	JQ011969.1
Grindelia pulchella	JQ011960.1
Grindelia pulchella	JQ011964.1
Grindelia pusilla	JQ011938.1
Grindelia procera	JQ011944.1
Grindelia prunelloides	JQ011952.1
Grindelia pygmaea	JQ011956.1
Grindelia revoluta	JQ011902.1
Grindelia revoluta	JQ011923.1
Grindelia robinsonii	JQ011924.1
Grindelia scabra var. *scabra*	JQ011929.1
Grindelia scorzonerifolia	JQ011970.1
Grindelia squarrosa	JQ011904.1
Grindelia squarrosa	JQ011905.1
Grindelia squarrosa	JQ011906.1
Grindelia squarrosa	JQ011912.1
Grindelia squarrosa	JQ011919.1
Grindelia squarrosa	JQ011934.1
Grindelia squarrosa	JQ011935.1
Grindelia squarrosa	JQ011936.1
Grindelia squarrosa	JQ011937.1
Grindelia stricta var. *platyphylla*	JQ011925.1
Grindelia stricta var. *platyphylla*	JQ011941.1
Grindelia stricta var. *stricta*	JQ011897.1
Grindelia stricta var. *stricta*	JQ011916.1
Grindelia stricta var. *stricta*	JQ011917.1
Grindelia subalpina	JQ011918.1
Grindelia tarapacana	JQ011961.1
Grindelia cf. tenella	JQ011986.1

Tree-based analysis

Asteraceae tree

A Bayesian phylogenetic approach bases itself on character information from the sequence alignment provided. The phylogenetic tree is constructed from the probability of a tree given the data and searches for trees for an assigned number of iterations. As the iterations go by, trees are scored, if a tree has a greater likelihood than the previous one it is replaced by this one and the previous one is discarded. Additionally, a model of sequence evolution is used as well such as the General Time-Reversible, which assumes all the rates of changes from a nucleotide to another are independent and are allowed to vary (Tavaré, 1986). Posterior probabilities, which are shown on the branches, measure the confidence of each assignment (Munch et al. 2008).

Bayesian analyses were run using MrBayes v. 3.2.2 (Huelsenbeck and Ronquist 2001) plug-in on Geneious 7.1.4 (Kearse et al. 2012) using the concatenated matK and rbcL data set of 2241 bp (matK: 811 bp, rbcL: 1430 bp).

The data set consisted of 166 sequences, including five outgroup species, six "Arnica" samples, two of which were *Arnica montana* (20055, 20056) and 154 accessions of Asteraceae found in the Río Grande Valley or representing those found in the area. Tables 2 and 3 indicate the source of each sequence. Of these 154 accessions matK and rbcL represented 146 and 147 respectively.

The General Time Reversible model of sequence evolution was used in the analysis. *Adenophora divaricata* served as the outgroup. A gamma rate variation with four Gamma Categories was used. The MCMC settings were as follows: Run of 5,000,000 generations, sampling every 1,000 generations. Four heated chains were used with a heated chain temperature of 0.2. The burn-in length was set to 1,500,000. The priors were unconstrained branch lengths: exponential (10) and shape parameter: exponential (10).

Subtrees:

Senecioneae:

The same parameters used for the Asteraceae family tree were employed in this subtree. ITS sequences were 581 bp, and consisted of the *Senecio arnaldii-Pseudogynoxys* clade of ITS sequences obtained from the GenBank PopSet (156753992) of Pelser et al. 2007, a GenBank accession of *Pseudogynoxys benthamii* (AF459958.1) and Arnica 20077, as indicated on Table 4. *Senecio arnaldii* was used as the outgroup.

Chrysopsidinae:

The same parameters used for the Asteraceae family tree were employed in this subtree. ITS sequences were 638 bp, and consisted of sequences from the PopSet of Teoh, Starr and Brewer unpublished (262233172), additional GenBank species in Chrysopsidinae and Arnica 20029 (Table 5). *Geissolepis suaedifolia* served as the outgroup.

Machaerantherinae:

The same parameters used for the Asteraceae family tree were employed in this subtree. ITS sequences were 644 bp, and consisted of from the GenBank PopSet of Moore et al. 2012 (358627872), selected sequences from the PopSet of Karaman-Castro and Urbatsch (94494713) and Arnica 20057 (Table 6). *Eurybia wastachensis* served as the outgroup.

Basic Local Alignment Search Tool

A BLAST search for nucleotides was conducted with the default settings to compare the results with those of the phylogenetic tree analysis. The sequences in Table 2 were used as query sequences (Arnica 20055, Arnica 20056, Arnica 20057, Arnica 20077, Arnica 20063, and Arnica 20029) in matK, rbcL, and ITS. The BLAST search results were recorded (Table 7).

CHAPTER III

Results

3.1 Resolving the location of unknown "Arnica" samples

The phylogenetic tree for the Asteraceae family (Figure 4) represented 5 subfamilies and 18 tribes and indicated that the four unknown "Arnica" species could be found in different parts of the tree indicating they were not *Arnica montana*. Arnica 20063 was placed within a group of *Trixis inula* samples of subfamily Mutisioideae and tribe Nassauvieae (Figure 5A). Arnica 20077 was located in subfamily Asteroideae and tribe Senecioneae (Figure 5B). Arnica 20029 and Arnica 20057 are located within subfamily Asteroideae and tribe Astereae (Figure 5 C, D). The true *Arnica montana* accessions of subfamily Asteroideae and tribe Madieae formed a clade with *A. angustifolia*, *A. dealbata*, *A. amplexicaulis* 179, and *A. mollis*.

3.2 Unknown Arnica sample 20063

The unknown "Arnica" sample was resolved on a branch belonging to subfamily Mutisioideae and tribe Nassauvieae (Bayesian posterior probability = 1) (Figure 4, Figure 5A). The branch consisted of two subclades one consisting of *Acourtia runcinata* 40 and *A. wrightii* 125 (BP = 1) and another, a series of conspecific accessions: Arnica 20063, *Trixis inula* 106, *T. inula* 130 and *T. inula* (BP = 1) which are sister to *T. divaricata* (BP = 1). A subtree was not constructed for this accession and Arnica 20063 was considered resolved as *Trixis inula*.

3.3 Unknown Arnica sample 20077

3.3 A---Bayesian Tree

Arnica 20077 was located within the clade of tribe Senecioneae (BP = 1) represented by several subclades, one containing *Packera tampicana* 10 and *Senecio ampullaceus* 12 (BP = 1) and the other subclade further divided into two additional subclades, one with Arnica 20077 and *Pseudogynoxys chenopodioides* (BP = 0.98)

and the other with *Emilia fosbergii* 109 and *E. sonchifolia* (BP = 1) (Figure 5 B).

3.3 B--- Subtree

The tree of subtribe Senecioninae (Figure 6) consisted of two sister clades. One clade contained *Misbrookea strigosissima, Werneria caespitosa, W. nubigena, Xenophyllum poposum,* and *X. dactylophyllum* (BP = 1). The second clade (BP = 0.99) consisted of *Dorobaea lacinata* sister to two subclades. The first subclade (BP = 0.89) contained *Jessea multivenia, Charadranaetes durandii, J. cooperi,* and *Talamancalia boquetensis* and *Garcibarrigoa telembina*. The second subclade (BP = 1) consisted of *Pseudogynoxys benthamii* sister to *P. hankei, P. chenopodioides*, and Arnica 20077 (BP = 0.57). The well-supported *Pseudogynoxys* clade indicated that the sample belonged in the genus *Pseudogynoxys*.

3.4 Unknown Arnica sample 20029

3.4 A---Bayesian Tree

Arnica 20029 was located within the strongly supported Chrysopsidinae subtribe (BP = 1) of tribe Astereae (BP = 0.68) (Figure 4). Subtribe Chrysopsidinae (Figure 5 C) consisted of *Heterotheca canescens* 82 and its sister subclade, a polytomy of Arnica 20029, *Croptilon rigidifolium* 23, *H. subaxillaris* 24, and *H. subaxillaris* 49 (BP = 0.72).

3.4 B---Subtree

The resulting tree consisted of four major clades and an unresolved *Pityopsis flexuosa* (Figure 7). The first clade contained four accessions of *Pityopsis graminifolia* var. *latifolia* (BP = 0.74) and two sister clades, one with two accessions of *P. ruthii* (BP = 0.88) and another (BP = 0.76) with three accessions *P. falcata* and a subclade of three accessions of *P. pinifolia* (BP = 0.98). A second clade (BP = 0.81) consisted of *Croptilon divaricatum* sister to a subclade of *Chrysopsis mariana* and *Chrysopsis gossypina* (BP = 0.99). A third clade (BP = 0.90) consisted of two

accessions of *P. oligantha* sister to a clade of two accessions of *P. aspera*, two accessions of *P. aspera* var. *adenolepis*, three accessions of *P. graminifolia* var. *aequifolia*, three accessions of *P. graminifolia* var. *tenuifolia* and two accessions of *P. graminifolia* var. *graminifolia* (BP = 0.99). The forth clade (BP = 0.99) consisted of two subclades one with *Croptilon rigidifolium* and *Noticastrum marginatum* (BP = 0.63) and another subclade with various *Heterotheca* species and Arnica 20029 (BP = 1). The subclade of *Heterotheca* was further divided into two subclades, one containing H. *fulcrata* and two accessions of *H. villosa* (BP = 0.98) and another with two *H. subaxillaris* in which the unknown Arnica 20029 was found (BP = 1). The results indicated Arnica 20029 was likely *H. subaxillaris*.

3.5 Unknown Arnica sample 20057

3.5 A---Bayesian Tree

Arnica 20057 was located within the Machaerantherinae subtribe (BP = 0.85) of tribe Astereae (BP = 0.68) (Figure 4). Subtribe Machaerantherinae (Figure 5D) consisted of *Xanthisma texanum* 60 and a sister clade (BP = 0.88) in which Arnica 20057 was found along with two strongly supported subclades. One subclade (BP = 0.99) consisted of *Xanthisma spinulosum* and *Grindelia squarrosa* and the other (BP = 0.99) consisted of *Isocoma coronopifolia* 140, *I. coronopifolia* 91, *I. drummondii* 141 and *Rayjacksonia phyllocephala 56*.

3.5 B--- Subtree

The subtree representing the subtribe Machaerantherinae consisted of two main *Grindelia* subclades, a North American and a South American. Arnica 20057 was located in the North American subclade (Figure 8). The North American subclade formed a polytomy of unresolved Arnica 20057, *G. greenmanii*, a subclade of *G. robinsonii* and *G. cf. tenella* (BP = 1), a subclade of two accessions of *G. oolepis* (BP = 1), a subclade of two accessions of *G. ciliata* and *G. adenodonta* (BP = 0.98), a subclade of *G. havardii*, *G.* scabra var. scabra, and *G. lanceolata* (BP = 0.98)

sister to a further subclade of *G. lanceolata*, a subclade of *G. grandifolia* (BP = 0.98) sister to the remaining North American *Grindelia* species (BP = 0.98) and a last clade of *Haplopappus setiger* and *G. inuloides* (BP = 0.98). The results indicated that Arnica 20057 belonged to the genus *Grindelia* and is of North American origin.

Basic Local Alignment Search Tool (BLAST) for sequence identity

When running BLAST searches for the unknown "Arnica" samples, ITS fared better than matK and rbcL. In five out of six species with ITS sequences, BLAST was able to identify the query sequence to the correct genus (Table 7) and the remaining species was identified to the correct subtribe using ITS sequences. Of the five samples identified to the correct genus three could be identified to the correct species and in the two additional cases the species of the sample was unknown, therefore it could not be verified. In the instance where the query sequence was identified to the correct subtribe, no sequence existed for that species in GenBank (*Trixis inula*). Among the parameters that a BLAST search includes are the query coverage and identity. As noted in Figure 1, a BLAST search can yield one of two things: a perfect match or a close match. In a perfect match, the sequence may be identical and be the actual species, otherwise it may be identical but be a different species. On the other hand, a close match may not be identical and still be the actual species due to intraspecific variation or it may not be identical and also be a different species. For instance, in the case of *Pseudogynoxys*, a BLAST search resulted in the following top three results: 1) *P. benthamii,* 2) *P. haenkei,* and 3) *Caxamarca sanchezii*. The sequences for *P. chenopodioides* which we included in our phylogeny and resulted closely related to our query sequences was not among the results on the BLAST search yet sequences for this species are available on GenBank.

A BLAST search of matK sequences correctly identified three out of six sequences to the genus level, one of which was identified to the species level as well (*Arnica, Trixis inula*). No matK sequences existed for *A. montana* in GenBank. In the

three remaining sequences the query was identified to the tribal level. Out of these three sequences, no sequence was available on GenBank in one case (*Grindelia*), however, in the two remaining cases a sequence matching the correct species or genus was available on GenBank but was omitted from the search results.

The BLAST results for rbcL identified the query sequences to the correct genus in five out of six cases and in three cases to the species level. In the remaining case where the species was identified to the tribal level, no sequence of the species or genus was available in GenBank (*Pseudogynoxys*). In two of the five cases mentioned previously where the genus or species was identified, the correct species was number 11 (*Grindelia*) or 59 (*Heterotheca subaxillaris*). In one instance, where the genus was identified correctly, the correct species was not included in the search results despite its availability in GenBank (*T. inula*).

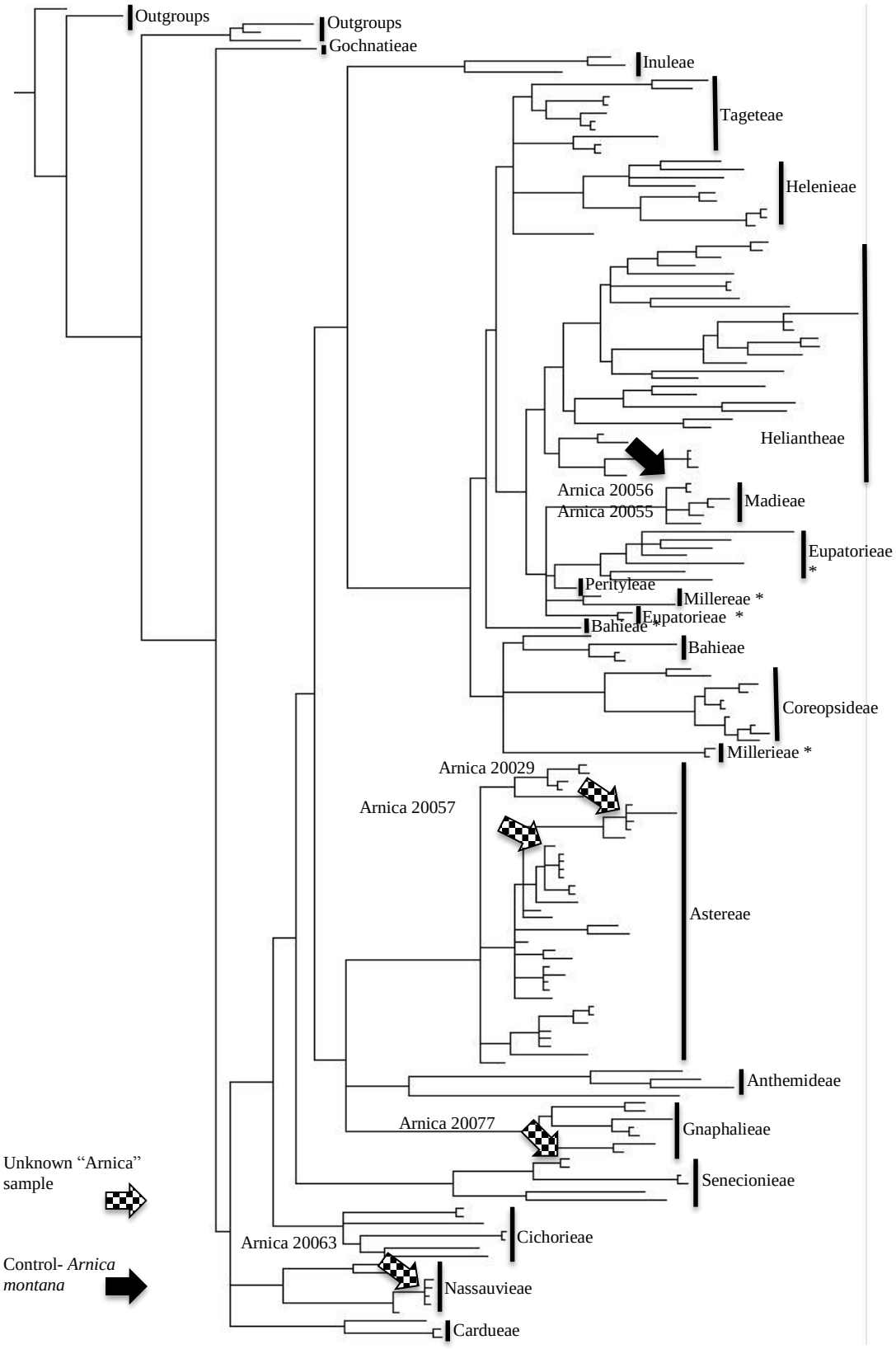
Outgroups
Outgroups
Gochnatieae
Inuleae
Tageteae
Helenieae
Heliantheae
Arnica 20056
Arnica 20055
Madieae
Eupatorieae *
Perityleae
Millereae *
Eupatorieae *
Bahieae *
Bahieae
Coreopsideae
Millerieae *
Arnica 20029
Arnica 20057
Astereae
Anthemideae
Arnica 20077
Gnaphalieae
Senecionieae
Cichorieae
Arnica 20063
Nassauvieae
Cardueae
Unknown "Arnica" sample
Control- Arnica montana

Figure 4 (Previous Page)
Phylogeny of Río Grande Asteraceae. Bayesian phylogenetic tree of concatenated matK and rbcL sequences. The checkered arrows represent unidentified "Arnica" samples. The black arrow indicates the control, or real accessions of *Arnica montana.*

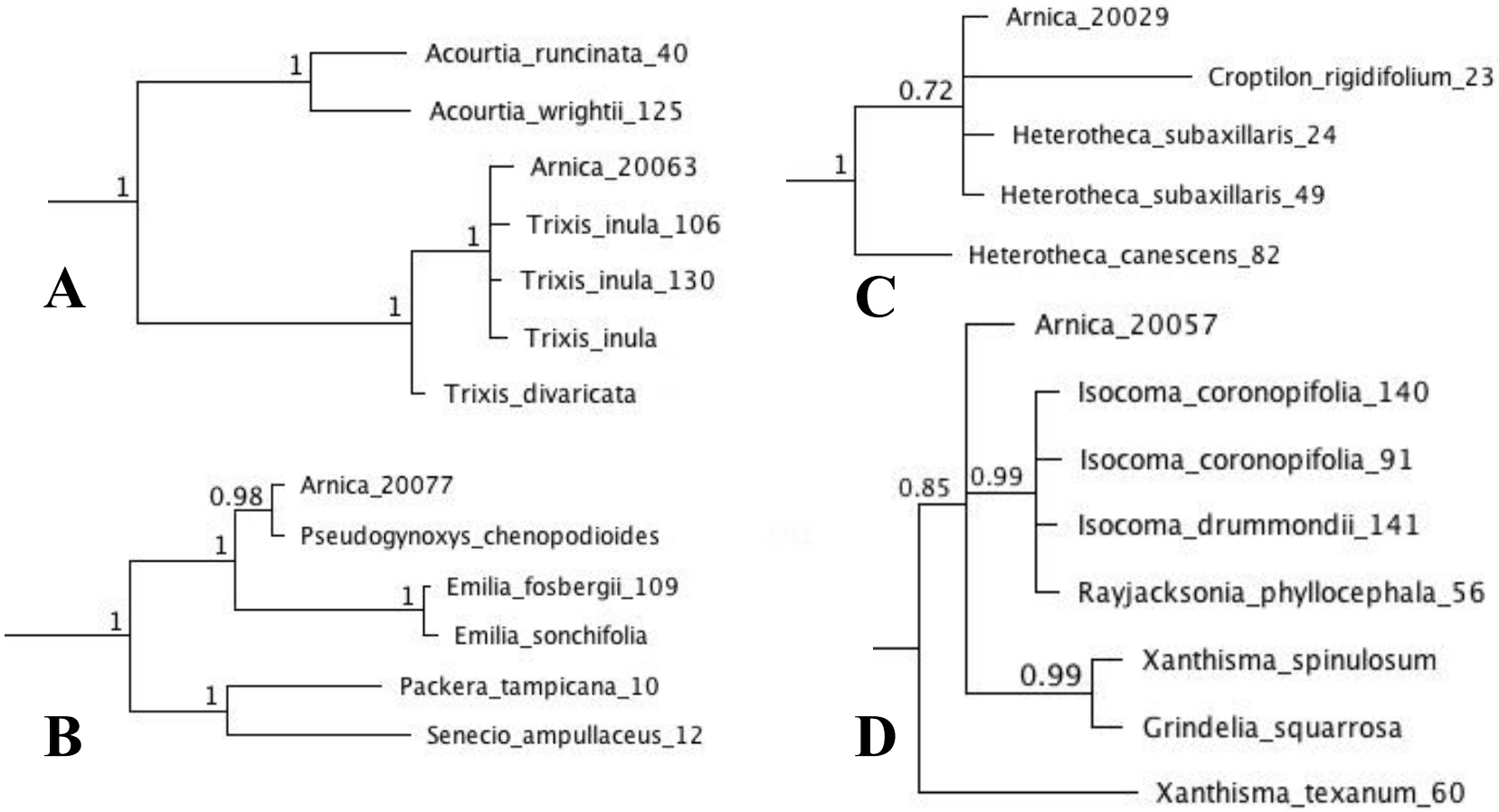

Figure 5
Placement of unknown "Arnica" species within a Bayesian tree. The above figures are enlarged portions of the Bayesian Asteraceae tree indicating the placement of "Arnica" species based on matK and rbcL data. A corresponds to tribe Nassauvieae. B corresponds to subtribe Senecioninae. C corresponds to subtribe Chrysopsidinae. D corresponds to subtribe Machaerantherinae.

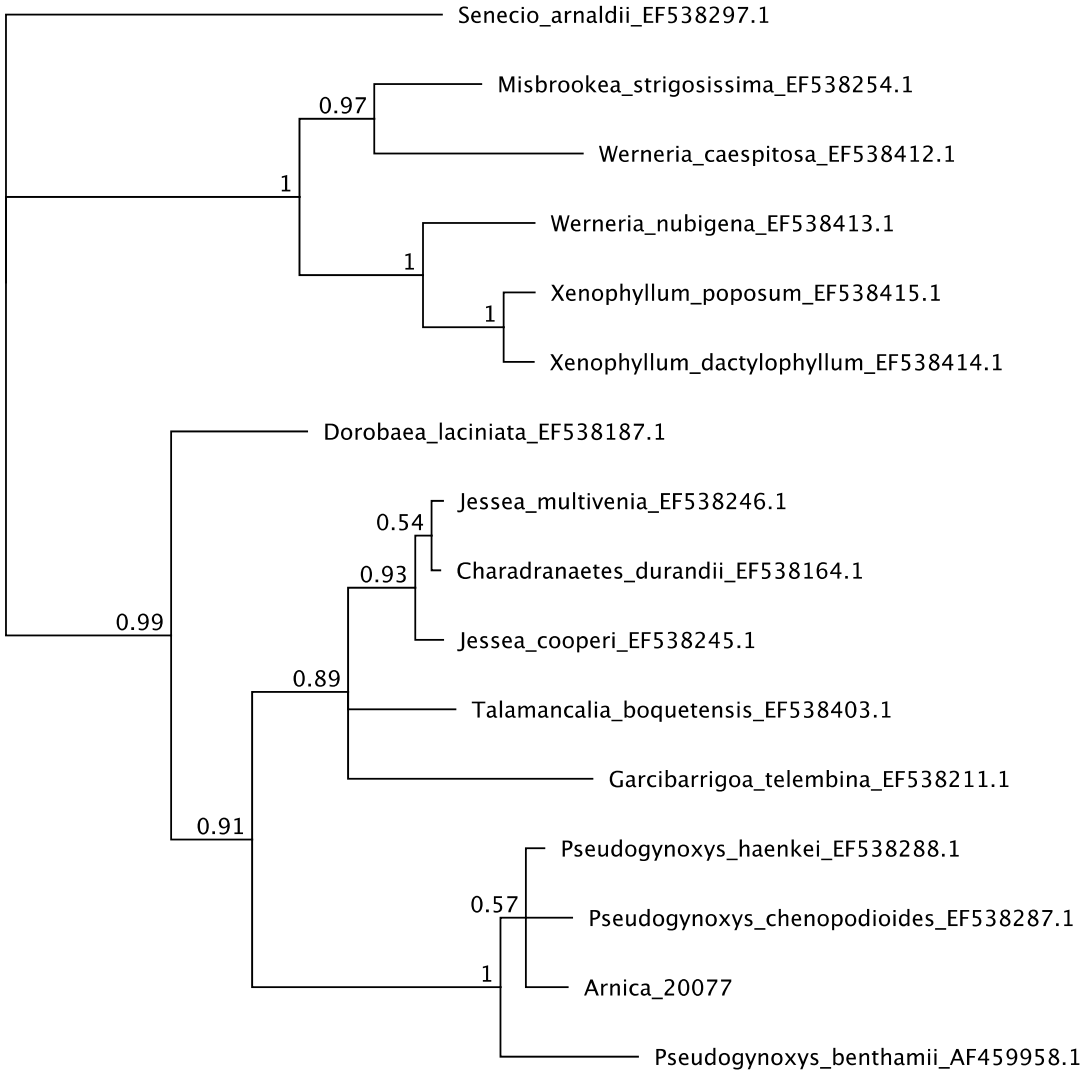

Figure 6
Placement of Arnica_20077 in Bayesian subtree of ITS data of subtribe Senecioninae. Bayesian posterior probabilities are above branches. The above figure is the enlarged area in which the unknown "Arnica" sample was resolved.

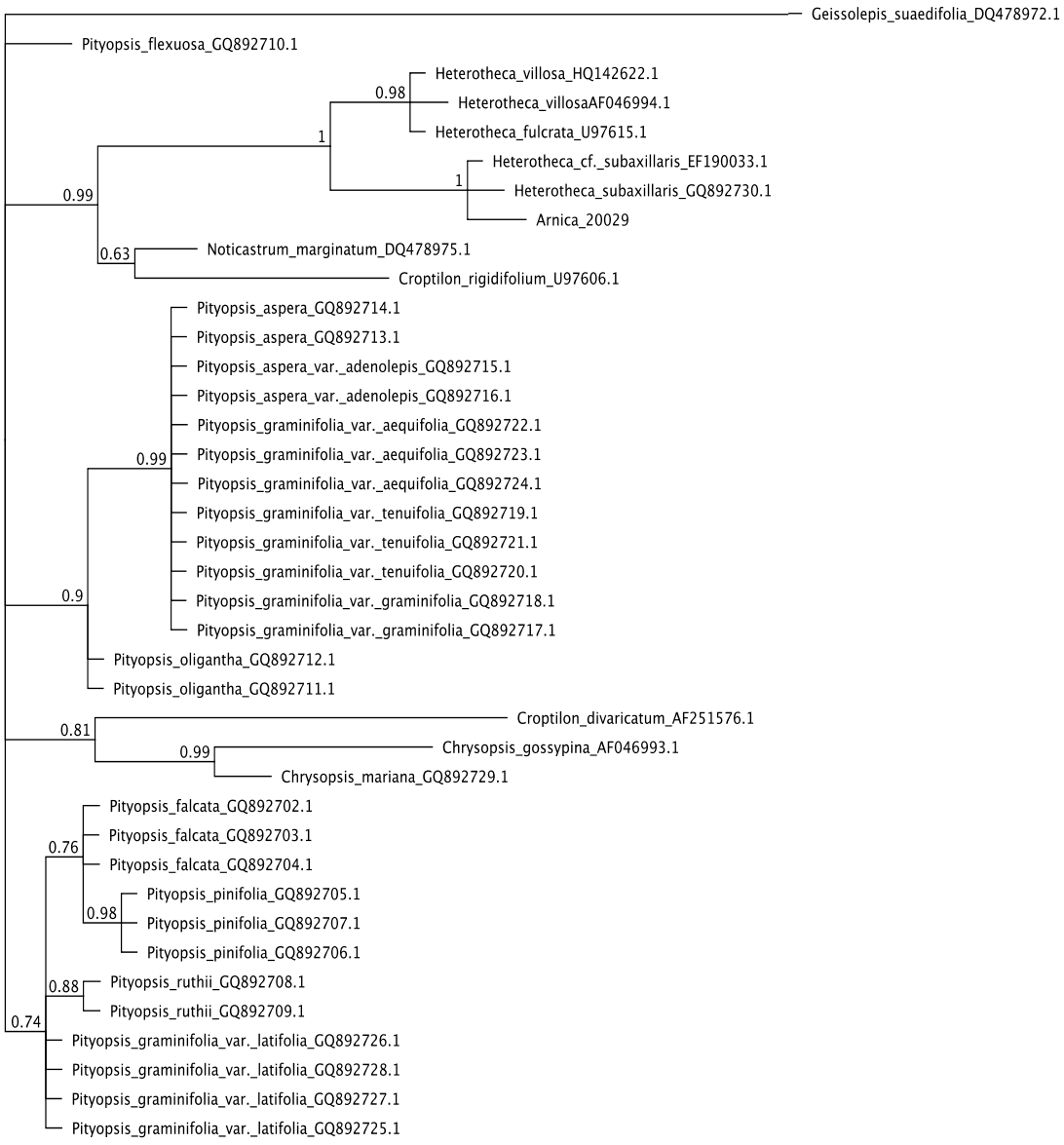

Figure 7
Placement of Arnica_20029 in Bayesian subtree of ITS data of subtribe Chrysopsidinae. Bayesian posterior probabilities are above branches. The above figure is the enlarged area in which the unknown "Arnica" sample was resolved.

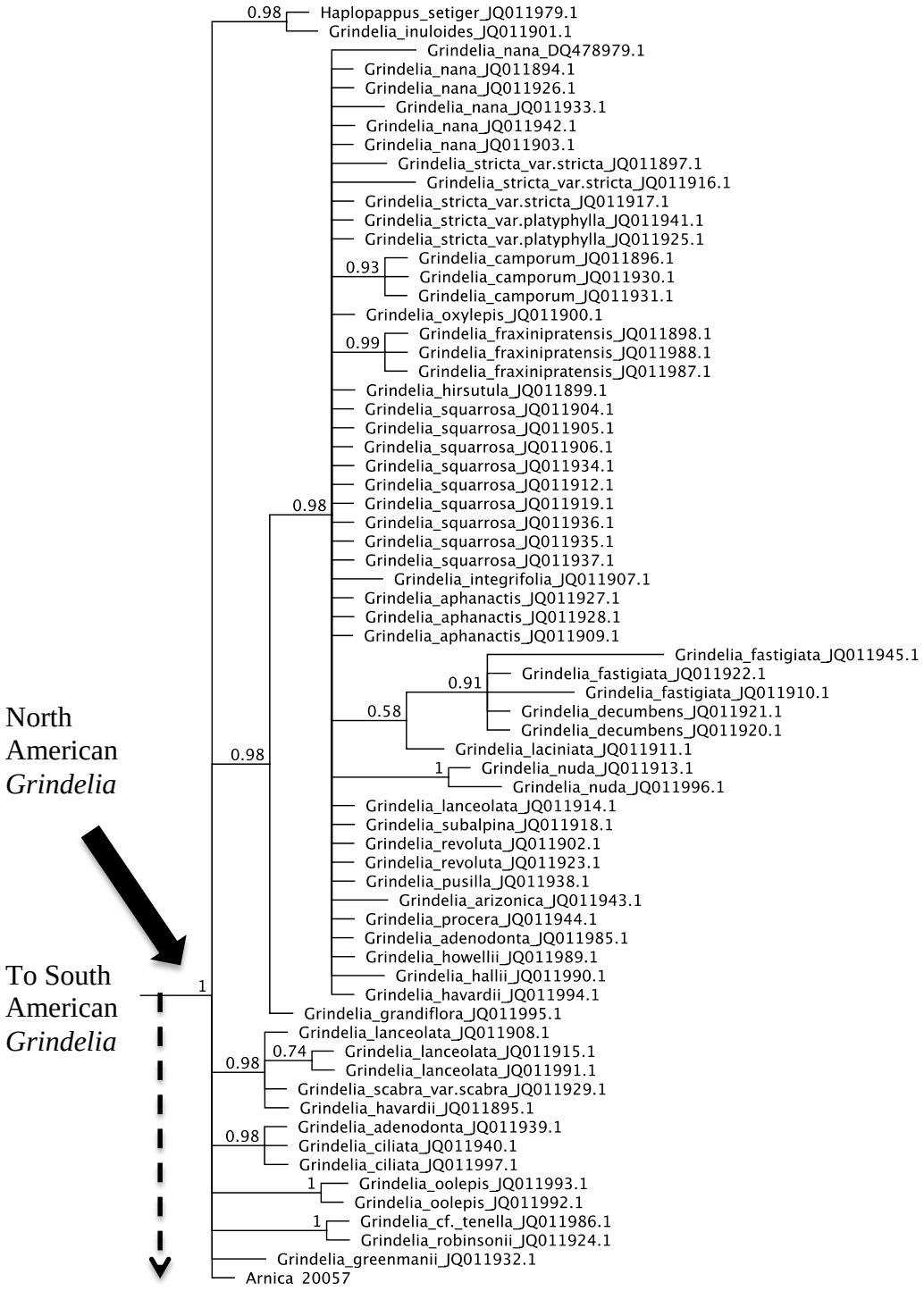

Figure 8
Placement of Arnica_20057 in Bayesian subtree of ITS data of subtribe Machaerantherinae. Bayesian posterior probabilities are above branches. The above figure is the enlarged area in which the unknown "Arnica" sample was resolved.

TABLE 7 Nucleotide BLAST Results

ITS	ITS	Max Score	Total Score	Query Coverage	E Value	Identity	Accession GB
20057 (*Grindelia*)	*Grindelia ciliata*	1149	1149	100%	0	99%	JQ011997.1
	Grindelia havardii	1149	1149	100%	0	99%	JQ011895.1
	Grindelia greenmanii	1146	1146	100%	0	99%	JQ011932.1
	Identified to genus, species unknown						
20029 (*Heterotheca subaxillaris*)	*Heterotheca cf. subaxillaris*	1157	1157	100%	0	99%	EF190033.1
	Heterotheca subaxillaris	1151	1151	100%	0	99%	GQ892730.1
	Heterotheca fulcrata	1101	1101	99%	0	99%	U97615.1
	Identified to genus and species						
20077 (*Pseudogynoxys*)	*Pseudogynoxys benthamii*	965	965	99%	0	98%	AF459958.1
	Pseudogynoxys haenkei	948	948	92%	0	99%	EF538288.1
	Caxamarca sanchezii	915	915	99%	0	96%	GU818509.1
	Identified to genus, species unknown						
20063	*Acourtia scapiformis*	475	475	96%	4,00E -130	80%	FJ979683.1
(*Trixis inula*)	*Moscharia solbrigii*	448	559	87%	8,00E -122	82%	EF530219.1
	Oxyphyllum ulicinum	436	547	89%	2,00E -118	81%	EU729343.1
	Identified to subtribe, sequence unavailable						
20055 (*Arnica montana*)	*Arnica cordifolia*	1249	1249	100%	0	97%	EF104922.1
	Arnica montana	1208	1208	90%	0	99%	HM032736.1
	Angiosperm environmental	1195	1195	98%	0	97%	FJ553475.1
	Identified to genus and species						
20056 (*Arnica montana*)	*Arnica cordifolia*	1245	1245	100%	0	97%	EF104922.1
	Arnica montana	1205	1205	90%	0	99%	HM032736.1
	Angiosperm environmental	1192	1192	98%	0	96%	FJ553475.1
	Identified to genus and species						

TABLE 7 Continued: Nucleotide BLAST Results

matK	matK	Max Score	Total Score	Query Coverage	E Value	Identity	Accession GB
20057 (*Grindelia*)	*Eurybia macrophylla*	1413	1413	98%	0	99%	KJ592941.1
	Euthamia graminifolia	1408	1408	98%	0	99%	KJ592944.1
	Solidago canadensis	1404	1404	99%	0	99%	EU749414.1
	Identified to tribe						
	No *Grindelia* matK in GenBank						
20029 (*Heterotheca subaxillaris*)	*Doellingeria sekimotoi*	1408	1408	99%	0	99%	AB262026.1
	Baccharis neglecta	1402	1402	99%	0	99%	EU385326.1
	Doellingeria rugulosa	1402	1402	99%	0	99%	AB262014.1
	Identified to tribe						
	H. subaxillaris: KJ772834.1						
	Omitted						
20077 (*Pseudogynoxys*)	*Senecio scandens*	1428	1428	99%	0	99%	HM989779.1
	Pericallis malvifolia subsp. *malvifolia*	1411	1411	99%	0	99%	HQ225971.1
	Pericallis malvifolia subsp. *malvifolia*	1411	1411	99%	0	99%	HQ225970.1
	Identified to tribe						
	***P. benthamii*:AF459983.1 Omitted**						
	***P. haenkei*: GU817510.1 Omitted**						
20063 (*Trixis inula*)	*Trixis divaricata*	1430	1430	99%	0	99%	EU385405.1
	Trixis inula	1386	1386	95%	0	100%	JQ586936.1
	Proustia cuneifolia	1382	1382	99%	0	99%	EU841351.1
	Identified to genus and species						
20055 (*Arnica montana*)	*Arnica mollis*	1439	1439	100%	0	99%	AY215764.1
	Arnica dealbata	1434	1434	100%	0	99%	AY215880.1
	Dyscritothamnus mirandae	1389	1389	100%	0	98%	AY215786.1
	Identified to genus						
	No *Arnica montana* matK in Genbank						
20056 (*Arnica montana*)	*Arnica griscomii* subsp. *frigida*	1380	1380	99%	0	99%	KC474109.1
	Arnica angustifolia subsp. *angustifolia*	1380	1380	99%	0	99%	KC474096.1
	Arnica mollis	1380	1380	100%	0	99%	AY215764.1
	Identified to genus						
	No *Arnica montana* matk in Genbank						

TABLE 7 Continued: Nucleotide BLAST Results

rbcL	rbcL	Max Score	Total Score	Query Coverage	E Value	Identity	Accession GB
20057 (*Grindelia*)	*Symphyotrichum novae-angliae*	2593	2593	100%	0	99%	GU817740.1
	Solidago canadensis	2549	2549	99%	0	99%	KM360988.1
	Erigeron tenuis	2540	2540	99%	0	99%	EU384973.1
	11-*Grindelia squarrosa*						
	Identified to genus, species unknown						
20029 (*Heterotheca subaxillaris*)	*Symphyotrichum novae-angliae*	2543	2543	98%	0	99%	GU817740.1
	Heterotheca villosa	2503	2403	96%	0	99%	JX848417.1
	Solidago canadensis	2499	2499	98%	0	99%	KM360988.1
	59- *Heterotheca subaxillaris* rbcL						
	Identified to genus and species						
20077 (*Pseudogynoxys*)	*Eschweilera simiorum*	1781	1781	93%	0	92%	JQ626117.1
	Sacciolepis indica	1421	1421	95%	0	86%	EF125137.1
	Caxamarca sanchezii	1321	2519	100%	0	99%	GU817745.1
	Identified to tribe						
	No *Pseudogynoxys* rbcL						
20063 (*Trixis inula*)	*Trixis divaricata*	2543	2543	98%	0	99%	EU385025.1
	Dolichlasium lagascae	2494	2494	100%	0	99%	EU384968.1
	Tarchonanthus camphoratus	2483	2483	100%	0	99%	KC589903.1
	Identified to genus						
	***Trixis inula*: JQ590725.1 Omitted**						
20055 (*Arnica montana*)	*Arnica montana*	2516	2516	99%	0	99%	KF602249.1
	Espeletia schultzii	2486	2486	100%	0	99%	KJ434459.1
	Arnica dealbata	2479	2479	97%	0	99%	AY215197.1
	Identified to genus and species						
20056 (*Arnica montana*)	*Arnica montana*	2516	2516	99%	0	99%	KF602249.1
	Espeletia schultzii	2486	2486	100%	0	99%	KJ434459.1
	Carramboa trujillensis	2475	2475	100%	0	98%	KJ434457.1
	Identified to genus and species						

CHAPTER IV
Discussion

Basic Local Alignment Search Tool (BLAST) for sequence identity

The BLAST searches indicated that a search with an ITS sequence provided the best results followed by rbcL and matK in subsequent order. This may be due to the fact that ITS is more variable. In addition, there is a greater quantity of GenBank sequences for ITS than there is for matK and rbcL which could be another reason a search with ITS yields better results. In most of the ITS searches the correct genus was identified by BLAST. However, there were two issues to address.

One of the issues was the inability of BLAST to include relevant sequences because sequences with greater coverage were given preference over those with shorter coverage. This is supported by Gemeinholzer et al. (2006), who found that complications can arise when ITS sequences are published as two separate sequences because longer sequences will have a preference over shorter sequences. The BLAST search for the ITS sequence of *Pseudogynoxys* illustrated this situation (Table 7). While ITS sequences for *P. chenopodioides* were available on GenBank, they did not appear in the BLAST results and were likely omitted due to a query coverage bias. Two of the available sequences for *P. chenopodioides* are of a shorter length than the BLAST search results, which would lead to a lower coverage and subsequently a lower score.

The second issue was the potential unavailability of a matching sequence in the database. Several sequences were either identified to the tribal level or to the genus level and a further investigation into GenBank revealed these sequences were not available in the database. One such case of this occurred in the BLAST search for *Grindelia* using a matK sequence. Results in the absence of a *Grindelia* yielded *Eurybia macrophylla*, *Euthamia neglecta* and *Solidago canadensis* (Table 7), which all belong to the same tribe and had a 99% identity and 98-99% coverage. In more conserved markers like matK and rbcL, search results where multiple genera each

have a 99% identity and similar coverage, it would be impossible for the researcher to discern the right species.

It is important to be aware of the assumptions made when identifying DNA sequences based on similarity such as through a BLAST search, which uses the GenBank Database. The assumptions are made are: the reference database is well sampled, the sequences in the database are identified and annotated correctly, and translating the comparisons into species names is standardized (Nilsson et al. 2006). Fungal species, Nilsson et al. (2006) found, were not correctly identified and or not annotated in the database. In the case of ITS sequences for fungi, they found that in fungi, there is a 20% chance of receiving a different species as the topmost match of BLAST results (Nilsson et al. 2006). Additionally, they found that in 8% of the cases even though the correct species was found in the topmost region, other insufficiently identified species hid them, similarly to what was found in this study (Nilsson et al. 2006). While some of these factors may apply specifically to fungi, others may apply to plant sequences as well.

In a study comparing BLAST results to phylogenetic nearest neighbor of a sequence, Koski and Golding (2001) found limitations in BLAST searches and highlight the importance of proceeding with caution when drawing conclusions based on these results. Sequence similarity such as that used in a BLAST search does not necessarily translate to close phylogenetic relatedness. Often times, the BLAST results did not correspond to the phylogenetic nearest neighbor of a sequence (Koski and Golding 2001). In some cases, the closest BLAST hit did not belong to the same domain at the query sequence, which would be problematic if one assumes the closest match corresponds to the correct identification (Koski and Golding 2001).

Based on the results of this study, an assumption that the first result yields the correct species is not always the case as illustrated in Table 7. Therefore, the recommendations when doing a BLAST search are to use as variable a region as possible, to pay close attention to identity, not only to coverage and to be cautious when drawing conclusions based on BLAST results.

Bayesian Phylogenetic Analyses

This study showed that a Bayesian phylogenetic approach was more effective than a BLAST search to identify unknown specimens.

One advantage of this method is that it is not based on sequence similarity like BLAST. Therefore, issues like a query coverage bias, which occurred in BLAST search should not be a problem in a Bayesian phylogeny.

Another advantage is the visual representation aspect, which one does not obtain by doing a BLAST search. By looking a the phylogenetic tree one can see where species fall whereas on a BLAST search one simply receives search results with no direct information as to how these results are related to each other and to the query sequence. In addition, in cases where the identification was not conclusive, additional subtrees helped with the identification process and may yield to more relevant results by narrowing species down. In this case more relevant information would refer to species that are more closely related to the unidentified sample as opposed to some of the BLAST searches where irrelevant information on unrelated results occurred.

One of the disadvantages of a Bayesian analysis, however, is that it requires a longer computational time. Nevertheless, despite the computational time, results are supported by statistics. Another disadvantage is that data omission would also affect a Bayesian phylogeny, although to a different extent. Still another disadvantage, is that with low posterior probabilities, one cannot have complete confidence in a particular clade.

Conclusion BLAST vs. Bayesian Phylogenetic Approach

By weighing the advantages and disadvantages and through careful consideration of the data a Bayesian phylogenetic approach was decided on for this study. Despite the computational time required for this process, a Bayesian approach is a statistical method that looks at characters instead of simply looking at similarity like BLAST does. BLAST is known to have several limitations as studies have found. Such limitations include coverage bias and providing search results based on

similarity alone (Gemeinholzer et al. 2006, Koski et al. 2001). A retrospective search using BLAST showed the limited ability to correctly identify species depending on the marker employed with markers like ITS being the better option. As found by Koski and Golding (2001), BLAST results are often not the nearest neighbor of a query sequence and the availability of close relatives in a database is a factor that influences this. By building phylogenetic trees and conducting BLAST search, the team found that the top BLAST results often did not correspond to the nearest neighbor of a query sequence and in some cases the BLAST result was from a species in a different domain of life (Koski and Golding 2001). Therefore, a phylogenetic approach is a better option when identifying unknown species.

Medicinal properties of *Arnica montana* and genera of "Arnica" samples

It was hypothesized that the samples of unknown "Arnicas" could be locally collected. While this cannot be confirmed, the evidence suggests that this may be likely, given that the species to which these samples were matched (*T. inula*, *H. subaxillaris*) or their genera (*Pseudogynoxys, Grindelia*) are present in the Río Grande Valley. An additional reason that would give support to this hypothesis is if these species or plants in the same genus posses medicinal properties and applications. In cases where no research has been conducted on the medicinal properties of a specific species, medicinal properties associated with that genus may indicate the need to research the species in the Río Grande Valley. The medicinal properties of these plants will be discussed along with their similarities in secondary compounds.

Arnica montana

Arnica montana, was the focus of this study as well as understanding its properties and if these were similar to those of its substitutes. A variety of chemical components and uses have been investigated in this species many of which are related to its medicinal use as an anti-inflammatory agent.

Arnica montana has a long history as a medicinal plant, especially in its native Europe where it is part of the 5th edition of the European Pharmacopoeia (Ganzera et al. 2008). It forms part of a genus of 29 circumboreal and predominantly montane species found in Asia, Europe and North America. Twenty-six of the species are found in North America with a high diversity in the western part of the continent (Wolf 2006). Specifically, *A. montana* is found in alpine meadows and sparse coniferous forests in the Pyrenees, the Alps, southern Russia, central Asia and Scandinavia (Clair 2010, Macêdo et al. 2004). The perennial plants of this genus have radiate or discoid heads with yellow to orange ray corollas and usually yellow disc corollas, and height ranges from 5-100 cm with basal or cauline leaves (Wolf 2006). Given its endangered status in its native habitat, *A. montana* is grown commercially in Europe and New Zealand among other countries as an alternative to collecting it from the wild (Roki et al. 2008).

A. montana is used to treat hematomas, dislocations, sprains, bruises, edema associated with fractures, and rheumatic muscle and joint complaints (Clair 2010). In Mexico the name "Arnica" is given to a variety of species that do not belong to the genus *Arnica*, but are used similarly, for example, *Grindelia inuloides*, *Helenium mexicanum*, *Heterotheca inuloides*, *Heterotheca leptoglossa*, *Neurolaena lobata*, *Tithonia diversifolia*, *Trixis angustifolia*, *Trixis californica*, and *Trixis radialis* (Waizel-Bucay and Cruz-Juárez, 2014, Estrada-Castillón et al. 2012*). Arnica montana* can be applied topically as an ointment or a poultice, or taken internally as a tincture. However, there is disagreement about its internal use with some arguing over whether it is safe at low doses, and others disapproving of any internal use altogether (Clair 2010). When applied externally, skin penetration behavior can vary depending on the type of preparation. When tested on a pigskin model, ointments exhibited a constant penetration rate of SLs whereas the rate decreased for gel preparations possibly due to drying (Wagner and Merfort 2007).

Flavonoids, SLs, and phenolic acids are the main constituents of *A. montana* (Šutovská et al. 2014, Wagner and Merfort 2007). The SLs found in *Arnica* are associated with its anti-inflammatory properties and the flavonoids with the

antimicrobial, antiphlogistic, and anti-rheumatic properties (Roki et al. 2008). The essential oil is responsible for *Arnica*'s antiseptic activity (Roki et al. 2008).

The SLs found in *A. montana* are of the 10α-methylpseudoguaianolide-type such as helenalin, 11α,13-dihydrohelenalin and their derivatives (Wagner and Merfort 2007). Their anti-inflammatory activity is attributed to the inhibition of the transcription factors NF-κB and NF-AT responsible for the transcription of genes that encode for inflammatory mediators (Ganzera et al. 2008, Klaas et al. 2002). Helenalin, however, is also responsible for skin irritation, gastroenteritis and internal bleeding in the digestive tract when consumed in large amounts (Šutovská et al. 2014). In humans, the consumption of a handful of leaves can be enough to induce severe headaches, miscarriage, delirium, convulsions, fatal poisoning due its action on the liver and central nervous system (Waizel-Bucay and Cruz-Juárez 2014). In Europe there are two known chemotypes of *A. montana*, each associate with greater quantities of one of two main SLs. Helenalin esters dominate in the central European chemotype whereas in the Spanish, 11α,13-dihydrohelenalin esters prevail (Wagner and Merfort 2007). Nevertheless, chemotypes of *A. montana* with high levels of helenalin have been found in heath lands in Spain by Perry at al. 2009. Knowing the content is important, because the two SLs can differ in their anti-inflammatory efficiency as well as their allergic side effects (Perry et al. 2009, Klaas et al 2002).

The phenolic and flavonoid components found in *A. montana* are believed to possess antioxidant properties and may protect the plant from ultraviolet damage. Among the constituents are the flavonoid aglycons: hispidulin, pectolinarigenin, 6-hydroxyluteolin 6-methyl ether and kaempferol and caffeic acid derivatives such as 3,5-dicaffeoylquinic acid (Nikolova et al. 2013). When tested on mouse fibroblasts, an ethanolic extract of *A. montana*, rich in the flavonoids and phenolic acid: quercetin, rutin, apigenin and chlorogenic acid, protected cells from the oxidative damage of hydrogen peroxide when applied as a pretreatment (Cracinescu et al. 2012). The types of flavonoids can differ between species of the genus, for example, the North American *A. chamissonis*, used as a substitute for *A. montana*, contains luteolin and luteolin-7-O-glycosides as dominant flavonoids whereas *A. montana*

contains quercetin and kaempferol (Roki et al. 2008). In addition, the environmental conditions can influence the phenolic content of a plant (Nikolova et al. 2013). For example, Spitaler et al. 2006, found that while there was no correlation between altitude and total flavonoid content, the concentration of phenolic acids such as caffeic acid derivatives and the ratio of 3',4'-dihydroxylated flavonoids to flavonoids without that substitution pattern increased with altitude. They believe the increased UV-B radiation experienced at higher elevations is one of the main factors leading to the biosynthesis of caffeic acid derivatives, which help with hydrogen peroxide scavenging and therefore protect the plant. Ganzera et al. 2008 on the other hand, found that both flavonoids and phenolic acids increased with altitude and temperature.

A study on antibacterial activity of medicinal plants against peridontopathic bacteria showed the methanolic extract of *A. montana* was effective against *Actinomyces spp*., *Eikenella corrodens*, *Peptostreptococcus spp*., *Porphyromonas gingivalis*, and *Prevotella spp*. (Iauk et al. 2003).

In *A. montana* the main chemical components are various types of flavonoids and pheolic acids and SLs which have exhibited antibacterial, anti-inflammatory and antioxidant properties. Our representation of this species in our data consists of a well-supported tribe Madieae consisting of *A. angustifolia*, 2 accessions of *A. montana* (*Arnica 20055, Arnica 20056*), *A. dealbata*, *A. amplexicaulis*, and *A. mollis.*

Trixis species

Among the unknown "Arnica" samples tested in this study, Arnica 20063 was identified as *Trixis inula* (Figure 4, Figure 5A). This genus belongs to the Asteraceae subfamily Mutisioideae, tribe Nassauvieae.

The genus *Trixis* contains 65 species, two of which are described in the Flora of North America (Keil 2006). *Trixis californica* is found in the southwestern United States and Northern Mexico. *Trixis inula* is found in Texas including the Río Grande Valley, Mexico, the West Indies, Central America and South America and grows in palm groves, roadsides, sandy sites, thickets and thorn scrub (Keil 2006). In North

America, the plant flowers from March to November and is known as Mexican trixis, hierba del aire, and tropical threefold (Keil 2006). Additionally, a variety of species, several of which have medicinal uses are found throughout Central and South America, in countries such as Argentina, Bolivia, Brazil, Chile, Colombia, Mexico and Paraguay (Agra et al. 2008, Degen et al. 2005, Estrada-Castillón et al. 2012, Martin-Granato et al. 2013, Hirschhorn 1981, Maldonado 2014, Martínez 2008, Pereira et al. 2005, Ribeiro et al. 1997, Rocha-Gracia et al. 2011, Trillo et al. 2010)

Plants in this genus are mainly used to treat inflammation of the eyes or conjunctivitis and uterine bleeding or menorrhea (Agra et al. 2008, Martin-Granato et al. 2013, Pereira et al. 2005). One such plant, *T. antimenorrhoea* (*T.divaricata*) derives its name from its use to treat uterine bleeding (Katinas 1996). Additional species are used to treat bruises, backaches, colds, diabetes, diarrhea, parasites, stomachaches, skin lesions, ulcerations, venereal disease as well as for feminine issues such as to hasten child birth and used as an abortive (Agra et al. 2008, Degen et al. 2005, Felger and Moser 1974, Maldonado 2014, Trillo et al. 2010). The parts of the plant employed depend on the use but can be the leaves, the roots, the stems or the entire plant.

Flavonoids and tannins believed to have antioxidant properties are major components in plants of the genus *Trixis* (Martin-Granato et al. 2013). Tannins, for example can exhibit anti-diarrheic and antiseptic properties when taken orally and externally they can act as antiseptics on the skin and mucosa to protect the underlying layers (Martin-Granato et al 2013). Due to their ability to precipitate proteins, tannins also have antimicrobial and anti-fungal effects (Monterio et al. 2005). SLs also play an important role as chemical constituents of *Trixis* and are characterized by having a Trixane skeleton (De Riscala 1989).

The Seri of Sonora, Mexico make a tea from the roots of *T. californica* to hasten childbirth (Felger and Moser 1974). In Mexico, El Salvador, Nicaragua, Panama and Colombia, *T. radialis* is used to ameliorate diabetes and venereal disease (Hirschhorn 1981). In Colombia and Panama both *T. radialis* and *T. frutescens* (*T. inula)* are used to heal wounds (Hirschhorn 1981). In Brazil, *T. antimenorrhoea*

known to have tannins and flavonoids is used to alleviate uterine bleeding and inflammation of the eyes (Martin-Granato et al 2013). When employed for uterine bleeding, a tea is made from the roots or fresh leaves. For eye problems such as ophthalmia and conjunctivitis, compresses made from the stems or leaves are used (Lorenzi and Matos 2008 in Martin-Granato et al. 2013). In Brazil as well as Argentina *T. divaricata* (*T. antimenorrhoea*), whose hydroalcoholic extract has shown anti-ulcerogenic properties when tested on rats, possibly due to its flavonoid and tannin content (Pereira et al 2005). In the Brazilian northeast, *T. divaricata* and its congener *T. vauthieri* are used as an abortive, to treat amenorrhea, or as a wash for conjunctivitis (Agra et al. 2008). Inhabitants of the Argentinian province of Córdoba, use the roots of *T. divaricata* to treat backaches (Trillo et al. 2010) or as an antidote (decoction) to be drunk, or as a wash or poultice made of the leaves or ground roots for insect, spider and snake bites (Martínez 2008). In Paraguayan markets, the roots of *T. nobilis* and *T. pallida* found under the name urusu katii are used as an antiparasitic (Degen et al. 2005).

Trixis antimenorrhoea and *T. vauthieri* have shown promising results against serious protozoan diseases such as leishmaniasis and Chagas disease, respectively (Maldonado 2014, Ribeiro et al. 1997). Chagas disease caused by *Trypanosoma cruzi* affects approximately 7 million people and if left untreated can damage the heart and the central nervous system (World Health Organization 2015). Ribeiro et al. 1997 found that the flavonoids sakuranetin and penduletin present in the extract of *T. vauthieri* exhibited tripanocidal activity against *T. cruzi* (Ribeiro et al. 1997). On the other hand, leishmaniasis is responsible for 1.3 million new cases and up to 30,000 deaths annually (World Health Organization 2015). This disease can cause skin lesions and in some cases can be fatal. In vitro studies done by Maldonado 2014 found the ethanolic extract of *T.* antimenorrhoea exhibited leishmanicidal activity against *Leishmania braziliensis* and *L. amazonensis*. The most potent compound was a sesquiterpenoid-based trixanolide derivative (9α-hydroxy-3-β-acetoxy-3-methylbutanoate trixikingolide-14-(3'-methylbutanoate) (Maldonado 2014).

Furthermore, a Mexican species of *Trixis* has shown anti-bacterial activities

on nosocomial infections (Rocha-Gracia et al. 2011). The extract of *T. silvatica* exhibited antibacterial properties against nosocomial infections of *Staphylococcus aureus* and coagulase-negative staphylococci resistant to Methicillin (Rocha-Gracia et al. 2011).

Several species of *Trixis* are employed for a variety of medicinal uses, the most prevalent being to reduce inflammation of the eyes and for uterine bleeding. While the anti-inflammatory activity was the property most similar to those *A. montana,* the chemical components seem to differ between the two genera. Both *A. montana and Trixis* possess SLs, helenalin and dihydrohelenalin in the case of *Arnica* (Wagner and Merfort 2007), and SL with a trixane skeleton in the case of *Trixis* (De Riscala 1989). Extensive literature was not available on the medicinal properties of *T. inula* specifically, however, it seems that *T. frutescens*, which was used to treat wounds, is a synonym for *T. inula* (Hirschhorn 1981, Katinas 1996). Given the variety of species in this genus used medicinally, it is possible that medicinal properties could potentially be attributed to this plant.

Pseudogynoxys species

The unknown "Arnica" sample that lead to this genus was Arnica 20077, identified as *Pseudogynoxys* (Figure 4, Figure 5B). This genus is part of the subfamily Asteroideae.

Pseudogynoxys is a genus of twinning and climbing vines in the tribe Senecioneae native to Mexico, the West Indies, Central and South America (Barkley 2006, Redonda-Martínez and Villaseñor-Ríos 2011). There are approximately 13 species in the genus comprising vines ranging in height from 50 to 500+ cm with orange to brick red corollas that flower year round (Barkley 2006). In the United States, *Pseudogynoxys chenopodioides* (Kunth) Cabrera is the only species reported by the Flora of North America and it is found as an introduced species in Florida where it persists after cultivation (Barkley 2006). In the Río Grande Valley it can be commonly grown, but does not seem to have escaped cultivation. In Mexico one can find *P. fragans, P. cummingii, P. haenkei* and *P. chenopodioides*, which is used

ornamentally (Romo de Vivar et al. 2007). While genera in Senecioneae are known to have toxic pyrrolizidine alkaloids, these have not been found in *P. chenopodioides* (Romo de Vivar et al. 2007). A study conducted on *P. chenopodioides* established the presence of a sequiterpene germacrene D derivative and quinols such as jacaranone (Mericli et al. 1989, Romo de Vivar et al. 2007). Jacaranone found in other species also in the tribe Senecioneae such as *Pentacalia desiderabilis* has been shown to be effective against the promastigotes of *L. chagasi*, *L. braziliensis* and *L. amazonensis* which are responsible for Leishmaniasis and Chagas disease (Morais et al. 2012). Several studies mention the leaves as the principal component used medicinally. In some cases the leaves of *P. chenopodiodes* are crushed and added to cream that is put on the facial cheeks to relieve toothache (Callejas-Chávez 2006). The leaves are also boiled to make a tea that is used for internal bruises or to wash wounds (Callejas-Chávez 2006, Pineda Manzano 2013.) The leaves can be roasted as well and applied to wounds for rapid scar healing (Pineda Manzano 2013). A comparative study conducted on the anti-inflammatory effects of *P. chenopodioides* aqueous extracts of 5 and 10 mg/kg vs. indometacin on Winstar rats with a carrageenan-induced paw edema found that while *P. chenopodioides* extracts had a lower anti-inflammatory activity than indometacin, they were less aggressive on the gastric mucosa (Alvarado et al. 2014).

In a phylogenetic study of Senecioneae with an ITS dataset, *Pseudogynoxys* was grouped within the clade *Senecio arnaldii-Pseudogynoxys* that was supported with a 98% boostrap value and posterior probability value of 1 (Pelser 2007). The clade consisted of two subclades one with *Charadranaetes, Dorobea*, *Garcibarrioga*, *Jessea*, *Pseudogynoxys* and *Talamancalia*, and the other with *Misbrookea*, *Werneria*, and *Xenophyllum* with *Senecio arnaldii* as the sister group of the clade (Pelser 2007).

Information available on medicinal properties of *Pseudogynoxys* was limited. It is also used to treat bruises like *A. montana*, and one of its chemical constituents has shown activity as an antiparasitic agent. which is also present in the genus *Trixis*. Given the limited data available, species in this genus warrant further investigation.

Heterotheca species

The unknown "Arnica" 20029 sample was identified as *Heterotheca subxaxillaris* (Figure 4, Figure 5C). *Heterotheca* belongs to the subfamily Asteroideae, and several medicinal properties have been associated with it.

Heterotheca is a genus of 28 annual or perennial plants in the tribe Astereae, subtribe Chrysopsidinae found in Canada, the United States and Mexico (Semple 2006, Brouillet et al. 2009). The Flora of North America mentions 17 species of the 28 (Semple 2006). Plants in this genus have yellow corollas and leaves with a camphor odor (Semple 2006). They are commonly known as goldenasters, camphorweed, or telegraph weed (Semple 2006). *Heterotheca subaxillaris* and *H. canescens* represent this genus in the Río Grande Valley (Richardson and King, 2011).

Heterotheca inuloides known as false arnica or Mexican arnica, has been widely studied for its medicinal properties. As the common name suggests, this plant is used similarly to *A. montana*. *Heterotheca subaxillaris* has also been studied to a lesser extent. The flowers of *H. inuloides* are used to treat contusions and wounds externally or inflammatory diseases, and fevers internally (Gené et al. 1998). *Heterotheca inuloides* is also used in post-operative treatment of thrombophlebitis (Coballase-Urrutia et al. 2011). Extracts of *H. inuloides* have demonstrated antioxidant activities (Coballase-Urrutia et al. 2010, 2011, 2013 Haraguchi et al. 1997, Ruiz-Pérez et al. 2014). Finally, this species has also demonstrated anti-microbial and anti-parasitic properties (Rodríguez-Chávez et al. 2015, Kubo et al. 1994, 1995).

The main components of plants in this genus are essential oils, flavonoids, polyacetylenes, sesquiterpenoids (ex. cadalenes), sterols, and triterpenoids (Delgado et al. 2001, Gené et al. 1998). The flavonoids and sesquiterpenoids have been studied in depth for their anti-inflammatory and anti-oxidant properties.

The aqueous extract of *H. inuloides* flowers demonstrated anti-inflammatory activity on a carrageenan-induced edema in rats (Gené et al. 1998). Furthermore, the butanol fraction of this extract was significantly more effective at inhibiting

inflammation than the ethyl ether and aqueous fractions; it inhibited dextran-induced inflammation and reduced abdominal constrictions in mice injected with acetic acid (Gené et al 1998).

In a different study Delgado and colleagues found the methanolic extract of the dried aerial parts of *H. inuloides* demonstrated a higher inflammatory inhibition than the acetonic extract when tested on 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced mouse ear edema. The most active compounds were dicadalenol, caryolan-1,9β-diol and quercetin and had a higher or similar percent inhibition of edema than the nonsteroidal anti-inflammatory drug indomethacin (Delgado et al. 2001).

A study by Gorzalczany et al. (2009) on various extracts of the aerial parts of the closely related *H. subaxillaris* var. *latifolia* tested on TPA induced ear edema found different results. Unlike Delgado et al. (2001), the dichloromethane extract was significantly anti-inflammatory whereas the petrol ether and methanol extracts were not. Anti-inflammatory activity on carrageenan-induced edema was not observed by any of the extracts. The fractions of the dichloromethane extract that exhibited anti-inflammatory activity had major flavonoid constituents such as santin, pectolinarigenin, 3,6-dimethoxy-5,7,4'-trihydroxyflavone and hispidulin. The anti-inflammatory activity could be a result of inhibition of the protein kinase C, the cyclo-oxygenase and/or the 5-lipoxygenase pathways of arachidonate metabolism (Gorzalczany et al. 2009).

Constituents of *H. inuloides* have also shown antioxidant properties. For example, Haraguchi et al. (1997) tested for sesquiterpenes 7-hydroxy-3,4-dihydrocadalin and 7-hydroxycadalin and the flavonoids quercetin and kaempferol. These authors found glycosides of both in *H. inuloides* and associated various degrees of antioxidant properties with these compounds. Flavonoids and sesquiterpenoids had potent scavenging activity on free radicals. Flavonoids exhibited the ability to scavenge enzymatically and non-enzymatically generated superoxide ions. Sesquiterpenoids showed antioxidative activity against linoleic acid autoxidation.

The polyphenols quercetin, D-chiro-inositol, and spinasterol found in abundance in the methanolic extract of *H. inuloides* showed the highest free radical

scavenging properties (Coballase-Urrutia et al. 2013).

Coballase-Urrutia et al. (2011) found that the methanolic extract of *H. inuloides* rich in flavonoids, and the acetonic extract rich in sesquiterpenoids exhibited hepatoprotective properties in rats exposed to carbon tetrachloride (CCl_4). Given the methanolic extract's greater effect, further research on the extract and on quercetin, one of the main components, suggests that the hepatoprotective capacity is associated with their antioxidant properties (Coballese-Urrutia et al. 2011).

In addition, the components of *H. inuloides* may have antimicrobial and anti-parasitic properties according to some studies (Kubo et al. 1994, 1995, Rodríguez-Chávez et al. 2015). The sesquiterpenoid 7-hydroxy-3,4-dihydrocadalin extracted from *H. inuloides* flowers showed bactericidal activity against the gram-positive bacteria methicillin-resistant *Staphylococcus aureus* (MRSA). The same compound (7-hydroxy-3,4-dihydrocadalene) as well as 7-hydroxycalamenene were active against *Giardia intestinalis*, the protist responsible for the gastrointestinal disease Giardiasis (Rodríguez-Chávez et al. 2015).

The genus *Heterotheca* consists of three sections, section *Heterotheca*, *Ammodia*, and *Phyllotheca* (Semple 2006). The presence or absence of ray florets, the morphology of cypselae and leaf traits are the characteristics used to determine the section in which species were placed (Semple 1996). In *Heterotheca* section *Heterotheca*, species are characterized by having radial heads and dimorphic cypselae (Semple 2006). Arnica 20029, which was identified in this study as *H. subaxillaris*, *H. grandiflora* and the medicinal species, *H. inuloides* constitute section *Heterotheca*. It is interesting to note that two species with medicinal properties are closely related and found in the same section of the genus. As far as medicinal use in this genus, species such as *H. inuloides*, and *H. subaxillaris*, exhibit similarities with *A. montana* in that they reduce inflammation and are applied on contusions and bruises. Like *A. montana*, they also contain flavonoids and sesquiterpenes.

Grindelia species

In the present study, Arnica 20057 was identified as a North American *Grindelia* (Figure 4, Figure 5D). Many species in this genus are known for their medicinal use, both in North and South America. The employment of this sample for medicinal purposes is therefore supported.

Several *Grindelia* species are part of the United States Pharmacopeia and the National Formulatory (Brinker 2006). The new world genus *Grindelia* belongs to the subtribe Machaerantherinae and the tribe Astereae and consists of annuals, biennials, perennials and subshrubs. The species have radiate heads of yellow to orange disc corollas, and yellow ray corollas but in some species have discoid heads (Strother and Wetter 2006). The leaves are basal and cauline, alternate and usually glabrous and gland-dotted (Strother and Wetter 2006). The number of *Grindelia* species varies from ca. 30 to 70 species depending on the source (Bone 2006, Brinker 2006, Steyermark 1934, Strother and Wetter 2006, Valant-Vetschera and Wollenweber 2007).

In North America they are found in the central and western regions and have been secondarily introduced to the east (Strother and Wetter 2006). The Flora of North America mentions approximately 30 species of *Grindelia* with descriptions for 18. In the United States, California is the most speciose area harboring 13 species (Brinker 2006).

In South America, ca. 25 species of *Grindelia* can be found from Peru to Argentina including Chile, Bolivia, Brazil, and Uruguay (Bartoli et al 2003). Argentina contains 15 species of *Grindelia*, 8 of which are endemic (Roitman1999). *Grindelia chiloensis*, and *G. boliviana* are among the South American species that have been studied for their medicinal properties.

Commonly known as gum-plants, and resin-weeds (Strother and Wetter 2006, Bertaccini et al. 2011), a principal characteristic of plants in this genus is the resinous exudates found on their leaves, stems, involucres (Hoffmann et al. 1984) buds, and flower heads (Brinker 2006). It is in this resin where the medicinal properties of this plant are found.

Grindelia robusta, *G. camporum*, and *G. squarrosa* are used medicinally and contain grindelic acid as a dominant component as well as 17-substituted homologues of grindelic acid (Brinker 2006, Timmermann et al.1983, 1985a,b, 1986, 1987). While these species have been widely studied for their medicinal use, species such as *G. microcephala* and *G. oolepis*, which are found in the Río Grande Valley, have not.

The traditional medicinal uses for various *Grindelia* species are vast. In North America, native people have used grindelias as an antitussive, expectorant, sedative, and anti-asthmatic (Bone 2006, Felter 1922, Krenn et al. 2009, El-Shamy et al 2000, Soares et al. 2006). More specifically, in California, the Native Americans used the resin from the plant to treat asthma and bronchitis (La et al 2010, Bone 2006). At the present time, *G. robusta* is used in pharmaceuticals as an antitussive and antiasthmatic (Kaltenbach et al. 1993, El-Shamy et al. 2000 in La et al. 2010). It is thought that the treatment of respiratory diseases may be attributed to the essential oils (Fraternale 2007). *Grindelia* relaxes the bronchi and is said to act by producing a primary increase of secretion on bronchial membranes followed by lessened expectoration and a decrease in breathing rate (Felter 1922). A fluid extract from the leaves and flowering tops of *G. robusta* can be used to treat asthma, colds, bronchitis with a harsh cough and pertussis (Brinker 2006).

Grindelia species have also exhibited anti-fungal and antimicrobial properties. In a study conducted on the antifungal effects of medicinal plants, the methanolic extract of the stem tissue of *G. robusta* inhibited activity of pathogenic and toxicogenic species *Fusarium oxysorum, F. verticillioides, Penicillium expansum, P. brevicompactum, Aspergillus flavus* and *A. fumigatus*. The authors of the study attributed the effects to the non-volatile terpenoids found in the resin (Zabka et al. 2010). Another study showed *G. boliviana* had antibiotic activity against *Streptococcus pyogenes* and *Staphylococcus aureus*. In the study, the essential oil and the alcoholic extract showed the greatest antibacterial activity. The authors believe the antibacterial activity may be due to the presence of diterpenes, flavonoids and polyacetylenes. Components in the essential oil to which the antibacterial activity is attributed are borneol, terpineol, α-pinene, and β-pinene (Vengoa-Figeroa and Tagle-

Carbajal 2000).

The resin in *Grindelia* species consists of components such as grindelane-type diterpenes, flavonoids, essential oil, acetylenes, saponins, tannins and phenolic acids (Krenn et al. 2009). In *G. robusta,* α-pinene, borneol, limonene and trans-pinocarveol are among the major constituents of the essential oil (Bertaccini et al. 2011; Fraternale et al. 2007). α-pinene has been reported as a major constituent in the essential oil of plants thought to have antimicrobial properties such as *Pinus patula* (Tomani et al. 2014). α-pinene, limonene and trans-pinocarveol are also present at varying levels in *Eucalyptus lehmannii*, which exhibited antimicrobial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* (Elaissi et al 2012). In regard to fungi, α-pinene and limonene have shown to have a synergistic antimicrobial effect on *Saccharomyces cerevisiae*, one of the main spoilage yeasts in fruit juices (Tserennadmid et al. 2011). In addition, limonene is a major chemical component of citrus oil, which is known for its antimicrobial activity. In a study conducted on *Grindelia* plants infected with *Candidatus Phytoplasma asteris*, the percentage of limonene and borneol present in the infected plants was higher than healthy plants, thus suggesting its antimicrobial activity (Bertaccini et al. 2011). Hydrophobic essential oil compounds function by increasing the permeability of the microbe's outer membrane causing it to leak cell contents and thus impact its growth (Tserennadmid et al. 2011, Hyldgaard et al. 2012). Once the outer membrane has been permeated, additional components in the essential oil can affect internal cell parts (Tserennadmid et al. 2011, Hyldgaard et al. 2012).

In addition, *G. robusta and G. camporum* are used to treat skin conditions such as, poison ivy and poison oak induced dermatitis, ulcers, eczema, and swellings, due to their anti-inflammatory and antibacterial properties (Brinker 2006 and Felter 1922). The flavonoid, quercetin-3-methylether, is among the chemical constituents thought to be partially responsible for the anti-inflammatory activity of *G. robusta* (Krenn et al. 2009). The anti-inflammatory potential of *G. robusta* on periodontitis is linked to its neutralizing or inhibiting effect on lipopolysaccharide induced inflammatory mediators and matrix metalloproteinases acting through the reduction

of nuclear factor κB p65 activation (La et al. 2010).

Like *A. montana, Grindelia* species have anti-inflammatory action, notably when applied to areas affected by poison ivy or poison oak induced dermatitis. Given the variety of medicinal uses in this genus it is likely Arnica 20057 has valid medicinal applications. However, given the limitations of the data, the sample could not be identified to the species level. This is of importance and in a future study additional markers may be used to identify the plant sample to the species level.

Medicinal Properties Analyzed

Genera of the plants sold as "Arnica" contain species of medicinal importance (Table 8). Their medicinal properties have been similar to those of *A. montana,* but additional properties in these plants have also been found. In cases where the medicinal properties of the local species have not been investigated, it could be an avenue of pursuit, which could lead to the discovery of viable substitutes. Below is a legend with the literature of where each property was found.

1. Wagner and Merfort 2007
2. Cracinescu et al. 2012
3. Nikolova et al. 2013
4. Nikolova et al. 2013, Roki et al. 2008
5. Nikolova et al. 2013
6. Cracinescu et al. 2012
7. Cracinescu et al. 2012
8. Nikolova et al. 2013
9. Clair 2010, Ganzera et al. 2008, Klaas et al. 2002, Pljevljakušić et al. 2013, Roki et al. 2008
10. Iauk et al. 2003, Roki et al. 2008
11. Clair 2010, Ganzera et al. 2008, Pljevljakušić et al. 2013
12. Clair 2010
13. Clair 2010
14. Clair 2010
15. Clair 2010, Roki et al. 2008
16. Keil 2006.
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Table 8 Plant properties summarized

Species	Number of Species	Main Chemical Components	Specific Flavonoids	Medicinal Properties	Properties similar to *Arnica montana*
Arnica montana		SLs (helenalin, 11α,13-dihydrohelenalin,)(1)	Apigenin (2), Hispidulin (3), Kaempferol (4), Pectolinarigenin (5), Rutin (6), Quercetin (7), 6-hydroxyluteolin 6-methyl ether (8)		Anti-inflammatory (9), anti-microbial (10), bruises (11), edema associated with fractures (12), dislocations (13), hematomas (14), rheumatic muscle and joint complaints (15)
Trixis	65 (16)	Flavonoids (17), SLs (Trixanes) (18), Tannins (19)	Penduletin (20), Sakuranetin (21)	Abortive (22), anti-protozoan (23), backaches (24), colds (25), diabetes (26), diarrhea (27), parasites, (28) skin lesions (29), stomachaches (30), ulcerations (31), uterine bleeding (32), venereal disease (33), wounds (34)	Anti-microbial (35), inflammation (36), bruises (37),
Pseudogynoxys	13 (38)	Sesquiterpenoids (Germacrene D, Quinols) (39)		Scar healing (40), toothache (41), wounds (42), possible anti-protozoan (43)	Anti-inflammatory (44), internal bruises (45)
Heterotheca	28 (46)	Essential oils (47), Flavonoids (48), Polyacetylenes (49), SLs (cadalenes) (50)	Hispidulin (51), Kaempferol (52), Pectolinarigenin (53), Quercetin (54), Santin (55), Spinasterol (56), 3,6-dimethoxy-5,7,4'-trihydroxyflavone (57)	Antioxidant (58), anti-parasitic (59), fevers (60), thrombophlebitis (61), wounds (62)	Anti-inflammatory (63), anti-microbial (64), contusions (65)
Grindelia	30-70 (66)	Diterpenes (67), Essential oils (68), Flavonoids (69), Grindelic acids (70), Polyacetylenes (71)	Quercetin-3-methylether (72)	anti-asthmatic (73), anti-fungal (74), antitussive (75), bronchitis (76), expectorant (77), pertussis (78), ulcers (79)	Anti-inflammatory (80), anti-microbial (81), periodontitis (82),

Sesquiterpenoids:

Sesquiterpenoids are secondary metabolites associated with protecting the plant against herbivory. Sesquiterpene lactones, which consist of three isoprene units and a lactone ring are associated with anti-inflammatory activity by inhibiting the transcription factor NF-κB (Bork et al. 1997). Several genera of "Arnica" substitutes were associated with medicinal properties of this type. In *A. montana*, the sesquiterpenoids found were helenalin and 11α,13-dihydrohelenalin, in *Trixis*, trixanolide derivatives (De Riscala 1989), in *Pseudogynoxys*, germacrene D derivatives and quinols (Mericli et al. 1989, De Vivar et al. 2007) and in *Heterotheca*, it was cadalenes (Delgado et al. 2001. Gené et al.1998). While specific action tied to sesquiterpenoids was not found for *Grindelia*, anti-inflammatory activity related to the inhibition of NF-κB was found (La et al. 2010).

Flavonoids:

Flavonoids are polyphenolic secondary metabolites found in plants that are responsible for some pigments, e.g. yellow floral colors, and are associated with antioxidant medicinal properties (Middleton et al. 2000). The flavonoids varied among genera, for example, those in *A. montana* included hispidulin, pectolinarigenin, 6-hydroxyluteolin 6-methylether, kaempferol, quercetin, rutin, and apigenin, (Nikolova et al. 2013, Roki et al. 2008) in *Trixis*, sakuranetin and penduletin (Ribeiro et al 1997), in *Heterotheca*, santin, pectolinarigenin, hispidulin, quercetin and kaempferol (Haraguchi et al. 1997, Gorzalczany et al. 2009) and in *Grindelia*, quercetin-3-methylether (Krenn et al. 2009). The flavonoids in common between *A. montana and Heterotheca* are hispidulin and pectolinarigenin. In *A. montana*, for example, the flavonoids are associated with protection from UV light and in *H. subaxillaris* they have exhibited anti-inflammatory activity (Spitaler et al. 2006, Gorzalczany et al. 2009). Kaempferol and quercetin are found in *A. montana*, *Heterotheca*, and have been shown to have anti-oxidant properties. In *Grindelia*, quercetin-3-methyether was associated with its anti-inflammatory activity (Krenn et

al. 2009). Information on the flavonoid content of the genus *Pseudogynoxys* was not found.

The previously mentioned genera have been associated with some or all of the following: antioxidant, anti-fungal, anti-inflammatory, anti-microbial, and or anti-parasitic properties.

Antioxidant:

Antioxidants help to reduce the injury to tissues that results from free radicals (Tuo et al. 2015). In *A. montana,* flavonoids and phenolics in that plant help protect it from ultraviolet damage (Spitaler et al. 2006). Flavonoids and tannins are believed to be the main antioxidant compounds in *Trixis* (Martin-Granato et al. 2013). Flavonoids such as quercetin and the sesquiterpenoids 7-hydroxy-3,4-dihydrocadalin, and 7-hydroxycadalin of *H. inuloides* have demonstrated antioxidant activity (Coballese-Urrutia et al. 2011, 2013, Haraguchi et al. 1997, Ruiz-Pérez et al. 2014). In *Grindelia*, the antioxidant properties are associated with the essential oil, which contains borneol, α-pinene, trans-pinocarveol, bornyl acetate and limonene (Fraternale et al. 2007). No information was found in regard to antioxidant attributes of the genus *Pseudogynoxys*.

Antifungal:

Antifungal activity has been shown in *Trixis* and *Grindelia.* In *Trixis* it has been attributed to the presence of tannins (Monteiro et al. 2005). In *Grindelia,* the antifungal properties are believed to be associated with terpenoids in the resin. The methanolic extract of species like *G. robusta* show inhibition of *Fusarium oxysorum, F. verticilloides, Penicillium exapnsum, P. brevicompactum, Aspergillus flavus,* and *A. fumigatus* (Zabka et al. 2010). *Grindelia squarrosa* has also shown to inhibit *Trichophyton mentagrophytes* (McChesney and Adams 1985 in Brinker et al. 2006). Interestingly, kaempferol, which has been shown to inhibit fungal cell division (Hwang et al. 2001) is present in *A. montana* and *H. inuloides*. No information was found for anti-fungal properties of *Pseudogynoxys*.

Anti-inflammatory:

Representatives from all four genera have anti-inflammatory properties to some extent, but mechanism of actions may differ. For example, *Trixis divaricata and T. vauthieri* are used for inflammation of the eyes; *P. chenopodioides* has been shown to have anti-inflammatory activity in carrageenan-induced paw edema tests; *H. subxaxillaris* and *H. inuloides* are applied on wounds and contusions much like *A. montana*; and *G. robusta* has shown anti-inflammatory activity on periodontitis and along with *G. camporum* are used to treat poison ivy and poison oak exposure (Agra et al. 2008, Alvarado 2014, Delgado et al 2001, Gené et al. 1998, Martin-Granato et al. 2013, Gorzalczany et al. 2009, and La et al. 2010). Similar to *A. montana, G. robusta* is able to attribute their anti-inflammatory activity to the inhibition of transcription factors (Ganzera et. al 2008, Klass 2002 and La et al. 2010).

Anti-microbial:

The methanolic extract of *A. montana* has shown antibacterial activity against peridontopathic bacteria species such as *Actinomyces spp*., *Eikenella corrodens, Peptostreptococcus spp*., *Porphyromonas gingivalis,* and *Prevotella spp*. (Iauk et al. 2003). Due to their abilities to precipitate proteins, tannins confer anti-microbial actions in *Trixis* (Monteiro et al, 2005, Martin-Granato 2013). *Trixis silvatica* has exhibited antimicrobial activity against nosocomial infections from *Staphylococcus aureus* and methicillin-resistant coagulase-negative Staphylococci (Rocha-Gracia et al. 2011). Similar antibacterial properties are also found in *H. inuloides* against methicillin-resistant *Staphylococcus aureus* attributed to its sesquiterpenoids (Kubo et al. 1994). Lastly, *Grindelia boliviana*, has been shown to inhibit *Streptococcus pyogenes* and *Staphylococcus aureus* due to properties of its essential oil and alcoholic extract, which contains diterpenes, flavonoids and polyacetylenes (Vengoa-Figueroa and Tagle-Carbajal, 2000). Anti-microbial studies of *Pseudogynoxys* were not found.

Anti-parasitic:

Genera such as *Trixis* and *Heterotheca* have shown to have anti-parasitic activities. In the case of *T. divaricata,* flavonoids and sesquiterpene lactones have been associated with anti protozoan activity against the agents responsible for leishmaniasis and Chaga's disease (Maldonado 2014, Ribeiro et al. 1997). The sesquiterpenoid 7-hydroxy-3,4-dihydrocadalene of *H. inuloides* was shown to act as a giardicidal agent (Rodríguez-Chávez et al. 2015). While not directly associated, the sesquiterpeneoid jacaranone found in *P. chenopodioides*, has been shown to be antiprotozoan against the agents responsible for leishmaniasis and Chagas disease when extracted from *Pentacalia desiderabilis* (Morais 2012). Anti-parasitic activity was not found for the remaining genera.

Conclusions

A variety of DNA barcoding studies have been conducted on herbal supplements and unidentified samples (Little and Jeanson 2013, Newmaster et al 2013, Rai et al. 2012, Stoeckle et al. 2011, and Spooner 2009). Product substitution, contamination and use of fillers have been documented in plant-based products (Newmaster et al. 2013, Stoeckle et al. 2011). This becomes a problem when the consumer's health is put at risk, whether by consuming a toxic substance or simply due to inappropriately labeled products of no value. For this reason, correct product labeling is of paramount importance. Products sold under a common name can lead to problematic situations where the consumer may not know what he or she consumed and subsequently faces health issues. By including a scientific name on a product label, the consumer should be assured of the product he or she is using. This would be important in the case of allergic reactions and possible intoxication. In this study the evidence suggests that the products were in fact substitutions, however, they seem to be legitimate replacements, which may have medicinal properties. This conclusion is made based on a comparative analysis of literature information, but not tested

comparatively in vitro or vivo. These types of studies are very much needed to better evaluate alternative sources of medicinal plants.

Two limitations were encountered in this study, the limited availability of DNA reference sequences and the lack of marker variability in closely related species. In the first instance, when constructing the subtrees for the specific clades, the availability of sequences in specific genera such as *Trixis*, *Heterotheca* and *Pseudogynoxys* was limited to a few species. In their study of barcoding commercial teas, Stoeckle and colleagues experiences a similar scenario when searching on GenBank. Furthermore, in some instances there is a disparity in the availability of sequences for the same species, for example, a matK sequences but no rbcL or vice versa. The second limitation faced was experienced especially in the case of *Grindelia*, where a polytomy of *Grindelia* species was obtained from the phylogenetic analysis. In a previous phylogenetic study of *Grindelia*, multiple markers were used which likely explain the improvement in species resolution (Moore et al. 2012). Therefore the recommendation for future studies, in order to overcome these obstacles, would be 1) to obtain more reference plant material, which is limited in real-life applications and 2) to add more markers to the study to improve resolution.

DNA barcoding confirmed that samples commonly sold as *Arnica* are not *A. montana*, but instead *Trixis inula*, *Heterotheca subaxillaris, Pseudogynoxys* spp. and *Grindelia spp.* The evidence seem to suggest that the samples may be substitutes for *Arnica montana* and have similar medicinal properties or they may be used for medicinal properties in addition to those of *A. montana*. Literature on the multiple genera to which the samples were assigned attribute antioxidant, anti-fungal, anti-inflammatory, anti-microbial, and or anti-parasitic properties to plants in these genera. Given the presence of species of all the genera identified in the Río Grande Valley, the possibility of the plants being locally collected is plausible. Additional studies on these specific plants and those that have not been explored but belong to the same genera would be an area of potential richness in respect to medicinal plants and warrants further investigation. While these plants may have otherwise been

considered “Arnica” and thought of as *A, montana*, this study’s findings showed the true identification of these plants, which may be economically useful. This study also brings to attention the importance of ethnobotanical research in the area and how vital it is that information from people using medicinal plants is translated into the literature in an accurate way without miscommunications or assumptions on the identity of medicinal plants.

CHAPTER V
Recommendations

In this study all unknown "Arnica" samples were identified at least to the genus level (*Trixis*, *Pseudogynoxys*, *Heterotheca,* and *Grindelia*) and in some cases to the species level (*Trixis inula*, *Heterotheca subxaxillaris*). However, among the limitations encountered were the lack of sequences and research articles regarding the species identified or their conspecifics and their medicinal components and properties. Ideally, all of the species in question would be identified to the species level. In order to do so, one would have to obtain plant material for all the species in the genera, develop barcodes and run subtrees with a more complete ITS data set. In the cases of species like *Trixis inula,* and *Heterotheca subxaxillaris* it would serve to have more confidence in the identification and in *Pseudogynoxys* it may help resolve the genus. If this procedure still did not help to identify the species, additional markers would be needed. Such is the case of *Grindelia,* where not enough resolution was received with ITS alone, the use of an additional marker would therefore be helpful. This would not only help to identify these species but could help increase the number of representatives in GenBank.

Working with such a diverse family as Asteraceae where more than 1600 genera, and 24,000 species exist (Lundberg, 2009) it is difficult for every genus or species to be represented in databases, let alone be represented for a specific marker. In addition, there are cases in which a sequence for a species is available for a marker, but not for another, thus making comparative studies more difficult. Therefore, by obtaining this information the amount of much needed sequences in GenBank can greatly increase. This as a consequence would further the goals of DNA barcoding and help with plant research in the future.

Lastly, a factor to be addressed is the need for more research to be conducted on the chemical and potentially medicinal properties of various species mentioned in this study. Literature searches on medicinal properties or specific chemical components of *Trixis inula* or *Pseudogynoxys* were a clear indication of this.

Through this research, therefore the possibility of discovering congeneric species of plants that share the same or similar medicinal properties.

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