Molecular phylogenetics and molecular dating of Chilean *Puya* (Bromeliaceae)

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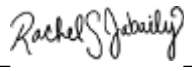

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Molecular phylogenetics and molecular dating of Chilean *Puya* (Bromeliaceae), Emma Stonesmyth and Rachel Jabaily Ph.D., Department of Organismal Biology and Ecology, Colorado College**,** May 1, 2020

Abstract

Puya is an understudied genus in the family Bromeliaceae. It is native to South America and is a characteristic feature of the high-Andean páramo. Its phylogeny is not well-resolved for many reasons, including that it has a poor collecting record and species have a high instance of hybridization. Molecular phylogenetics is a technique that uses DNA loci to reconstruct species phylogenies. The molecular phylogeny of *Puya* has only been reconstructed in two studies previously. In these studies, chloroplast DNA yielded a topology that was incongruent with the nuclear DNA topology and suggested an interesting history of introgression and chloroplast capture among the most ancestral lineages. The purpose of this thesis is to re-test the existing hypothesis with new molecular data and expanded sampling of the taxa from Chile. Polymerase chain reaction was used to amplify an additional nuclear locus, g3pdh. This locus was Sanger sequenced to reconstruct the phylogeny of the Chilean *Puya* and place them within the broader genus. This phylogeny was used to critique and expand the hypothesis of introgression and chloroplast capture, and to place the evolutionary history of the Chilean *Puya* within geological time through the process of molecular dating. Results found species within a previously hypothesized blue-flowered clade placed into two groups rather than one, and identified a novel clade of yellow-flowered Chilean species with other, more northern-Andean taxa. This new clade was named the Elevational Disjunct clade. Additional nuclear loci will be required to more fully explore the relationships between Chilean *Puya* and the rest of the genus. Including more collections within the northern Andes might better place the yellow-flowered Chilean taxa and allow for testing of an intriguing biogeographic history.

Key Words: Phylogenetics, *Puya*, Bromeliaceae, Chile, Incongruence, Molecular dating, Elevational Disjunct clade, Biogeography

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Introduction

In molecular systematics, DNA sequences are analyzed to determine relatedness among taxa. As lineages evolve, base-pair changes occur in the genome that accumulate over time. Using an appropriate locus, differences in genetic sequences can be reconstructed to represent evolutionary history, visualized as phylogenetic trees. To gain greater confidence in a particular phylogenetic topology, and to test alternate hypotheses about the topology, various hypothesis testing statistical analyses are used. The phylogenetic tree also serves as a framework on which other analyses are based. The tree can be time-calibrated using dated fossils as anchor nodes so the length of branches between nodes represent time. When matched to geologic events, molecular dated phylogenies point to potential causes of lineage splitting events and can be used to reconstruct the biogeographic history of a lineage. Habitat ranges of extant taxa, mapped onto a phylogeny and statistically analyzed, allow ancestral habitat ranges to be estimated to see where lineages originate and where hotspots of diversification existed. Another use for phylogenies is character reconstruction. It is a similar process to biogeographic analysis in which extant character states are mapped to estimate backwards what ancestral character states probably were.

Phylogenies are increasingly used to understand intricate evolutionary processes as well, the kind that are only observed when multiple different genetic loci are analyzed across the same group. Incongruences between loci are not simply problems to be overcome, they are data that

show different perspectives on the species tree and hint at the complex evolutionary processes at play. This thesis is a follow-up to (Jabaily & Sytsma, 2010), a molecular phylogeny of a genus in which the chloroplast and nuclear phylogenies were incongruent. I will test their hypothesis with new data in order to understand the reticulate evolution that led to the formation of the subject species.

Background of Bromeliaceae and *Puya*

The clade examined in this paper traditionally belongs to the genus *Puya* Molina. *Puya* is an important genus in the family Bromeliaceae (order Poales). Bromeliaceae is a neotropical monocot family characterized by a rosette vegetative morphology. It is best known for its epiphytes which densely cover tree branches in lowland tropical and cloud forests, but includes terrestrial plants too. Some are tank-forming and provide key habitat for insect larvae, and frog eggs and tadpoles. Members of the genus *Puya* share this rosette vegetative morphology. Their defining characters are petal spiraling after anthesis and leaf blades that are never contracted at the base (Smith and Downs, 1974). Most are iteroparous (repeated sexual reproductive events per lifetime), but some have evolved semi-semelparity and one species, *P. raimondii*, is fully semelparous (one sexual reproductive event in a lifetime). *Puya* live in the Andean mountains, from northern Venezuela and Colombia to central Chile, with two species in Panama and Costa Rica as well. They occur from sea level to over 4500 m. Most of the low elevation species occur in Chile. Some *Puya* are hummingbird pollinated (Hornung-Leoni et al., 2013); however, pollination in *Puya* is almost entirely unstudied.

Puya was originally placed in subfamily Pitcairnioideae based on the shared morphological features of winged seeds and dry fruits (Harms 1930; Smith and Downs, 1974). But based on subsequent chloroplast (cpDNA) phylogenetic analysis it was later made the only member of the subfamily Puyoideae, sister to the fleshy fruited Bromelioideae and apart from the remainder of the Pitcairnioideae subfamily (Escobedo-Sarti et al., 2013; Givnish et al., 2007; Givnish et al., 2011). *Puya* contains two subgenera: *Puya* and *Puyopsis*. The eight species in subgenus *Puya* are united by sterile inflorescence tips which are thought to serve as perches for pollinating hummingbirds. The species in subg. *Puya* are *P. alpestris, P. berteroniana, P. boliviensis P.*

castellanosii, P. chilensis, P. gilmartiniae, P. raimondii, and *P. weddelliana*. The remaining species, numbering over 210, are in subg. *Puyopsis* and have fertile flowers for the entire length of the inflorescence branches. These subgenera were established by Smith and Downs (1974) and upheld in a morphological and cladistic analysis by Hornung-Leoni and Sosa (2008). However, both genera were shown to be non-monophyletic based on molecular evidence by Jabaily and Sytsma (2010).

Puya contains over 218 species (Butcher and Gouda, 2019), with new species continuing to be described (Janeba, 2017; Trevino-Zevallos et al., 2019). Species concepts in *Puya* are not robust due to lack of information about most taxa. There is a poor collecting record and holotype specimens for many species are incomplete. In addition, some species encompass wide morphological variation. Most species are described as narrow endemics to mountain valleys, or high elevation Andean habitats like the páramo. Hybridization is known to occur (Schulte et al., 2010) and could potentially occur frequently as a result of minimal postzygotic barriers (Jabaily, pers. obs., 2009). Hybridization is certainly known to occur in the broader family, even between genera (Palma-Silva et al., 2011; Wendt et al., 2000; Zanella et al., 2016).

Background of Chilean *Puya* taxa

There are seven distinct, species-level taxa recognized in Chile. They are grouped into "Blue" *Puya* and "Yellow" *Puya* clades following Jabaily and Sytsma (2010). Blue *Puya* include *P. coerulea* and *P. venusta*. These species are united by their blue/purple floral petal color and elongated, tubular flower shape. *P. coerulea* is divided into four varieties: var. *violacea*, var. *monteroana*, var. *intermedia*, and var. *coerulea* (Smith and Downs, 1974). Yellow *Puya* include *P. chilensis* and *P. gilmartiniae*. These species are united by their yellow flower petal color, and shorter, more open flower shape. Their leaves are glabrous on the abaxial surface. *P. alpestris* and *P. berteroniana* are possible homoploid hybrid species between Yellow and Blue *Puya* and share a similar appearance (Jabaily & Sytsma, 2010). *P. alpestris* has blue flowers and *P. berteroniana* has blue-green flowers. Their leaves both have dense, white, appressed scales on the abaxial surface. Their flower color is reminiscent of Blue *Puya*, but they are shallower and

have a wider diameter, which is similar to Yellow *Puya*. *P. alpestris* is generally smaller although these taxa are very difficult to tell apart in the field. *P. boliviensis* is another species that displays characteristics of both Blue and Yellow *Puya*. It has yellow or yellow/green flowers with petals that form a tube that opens near the tip and leaves that become glabrous on the abaxial surface (Jabaily and Sytsma, 2010; Smith and Downs, 1974).

Zizka et al. (2013) revised the taxonomy within Chilean *Puya*. Based on field observations of morphology and habitat range of the Chilean *Puya*, and a review of specimens and the literature, they assert that *P. berteroniana* is not a species, but a recurring hybrid. They rename it *P. x berteroniana.* They do not predict what the parent species are, but based on morphology I predict that it is a hybrid between *P. alpestris*, a potential hybrid itself, and a member of Yellow *Puya*, and include it in our hypothesis (Fig. 1, step 3). However, there is uncertainty here as Zizka et al. found *P. x berteroniana* living in sympatry with *P. alpestris* subsp. *zoellneri* and *P. venusta,* not *P. alpestris.* Zizka et al. uphold the four varieties of *P. coerulea* from Smith and Downs (1974) and they add two subspecies of *P. alpestris*- *P. alpestris* subsp. *alpestris* form the southern populations, and *P. alpestris* subsp. *zoellneri* form the northern population, which they determine has sometimes been misidentified as *P. x berteroniana*.

Figure 2. Photos of some Chilean *Puya* species. From Left to Right: Top: *Puya gilmartiniae, P. coerulea, P. venusta.* Bottom: *P. coerulea, P. alpestris, P. chilensis*.

Puya alpestris occurs from Coquimbo (formerly the political district "Region IV") to Araucanía (formerly Region IX), from sea level to 2000 m in rocky habitats ranging from arid to humid, including the sclerophyllous forest of the Mediterranean-type climate. *Puya boliviensis* occurs only in Antofagasta (formerly Region II) from sea level to 670 m in rocky, arid areas. *Puya chilensis* occurs from Coquimbo to Ñumble and Bío Bío (formerly Region VIII) (abundant in Coquimbo and Valparaíso (formerly Region V) from sea level to 900 m in rocky habitats. *Puya gilmartiniae* occurs only in Coquimbo between 50 and 520 m in costal scrub vegetation. *Puya coerulea* occurs from Coquimbo to Araucanía from sea level to 2200 m in rocky arid, to semiarid habitats. *Puya venusta* occurs in Coquimbo and Valparaíso between 5 and 250 m in rocky areas, growing in dense stands (Zizka et al., 2013).

Phylogenetics

In 2010, Jabaily and Sytsma published molecular phylogenies of *Puya* taxa representative of the geographic range of the genus using three chloroplast regions (*matK, trnS-trnG,* and *rps16*) and one nuclear region (PHYC). The chloroplast loci and nuclear locus phylogenies are in general agreement regarding distinct northern Andean and central Andean *Puya* sub-clades, but are incongruent with respect to the seven taxa endemic to Chile. "Core" *Puya* (the term for non-Chilean taxa *sensu* Jabaily and Sytsma, 2010) are found throughout the Andes and a clear northern Andean clade was identified, but little phylogenetic structure was found in the taxa belonging to the central and southern Andes, outside of Chile. Chilean taxa are geographically isolated from Core *Puya* species. They are located in lowland Chile, isolated by the Atacama Desert in the north and the high, southern Andes in the east, which are generally devoid of *Puya*. *Puya* commonly grow at high elevation but neither the Chilean taxa nor their nearest *Puya* neighbors occur in the Andes in eastern Chile.

In the cpDNA phylogeny by Jabaily and Sytsma (2010), the Chilean taxa form a clade with the exception of the northernmost taxon, *P. boliviensis,* which is located in the fog-fed regions of the Atacama Desert. This "Chilean *Puya*" clade (excluding *P. boliviensis*) forms a polytomy with Bromelioideae and the remainder of *Puya* (Core *Puya*). Givnish et al., 2011 also found evidence

that the Chilean group is sister to all the rest of *Puya*. With this evidence, Chilean *Puya* occupies a putative basal position within the genus, calling attention to the significance of Chile as a biogeographical region. This gains greater significance with the fact that the southern temperate region of South America is biogeographically distinct from the northern tropical region. Many taxonomic clades have distributions within, but not between, the two regions (Apodaca et al., 2015).

In their eight-locus plastid phylogeny spanning all of Bromeliaceae, Givnish et al. (2011) corroborate the cpDNA topology by Jabaily and Sytsma (2010), finding *Puya* to be non-monophyletic, with Chilean *Puya* forming a separate clade. They sampled eight *Puya* species, three of which are Chilean taxa: *P. alpestris*, *P. venusta*, and *P. chilensis*. In their Maximum Likelihood (ML) and Bayesian Inference (BI) cpDNA phylogenies the Chilean taxa form a clade that falls sister to all other *Puya* plus Bromelioideae. However, in the ML phylogeny, the branch between the two separate lineages of *Puya* is very short, so it is not strong evidence for the non-monophyly of *Puya.* The BI phylogeny finds high support for the two separate *Puya* lineages. In contrast, the maximum parsimony (MP) phylogeny from Givnish et al., which uses the same chloroplast loci, places the Chilean taxa in a monophyletic *Puya* clade, still sister to all other sampled *Puya*. It is challenging to phylogenetically interpret very short branches from the minimal data separating Bromelioideae from *Puya,* and Chilean *Puya* from Core *Puya*; and morphologically, the non-monophyly of *Puya* is not supported because Bromelioideae taxa share a distinctive fleshy fruit not found in *Puya.* So the non-monophyly of the genus *Puya* is not currently a serious hypothesis.

In the nuclear PHYC phylogeny by Jabaily and Sytsma (2010), *Puya* resolves as monophyletic and the taxa from Chile form two clades, united within themselves broadly by flower color. This is in contrast to the cpDNA topology. Yellow *Puya* is sister to Core *Puya* and Blue *Puya* is sister to the remainder of *Puya* (Yellow *Puya* plus Core *Puya*) (Jabaily and Sytsma, 2010).

Since Jabaily and Sytsma (2010), others have used the gene PHYC for phylogenies in Bromeliaceae and found interesting results. Florian Krapp and colleagues used the gene PHYC to build a phylogeny of *Dyckia* (Bromeliaceae) which included two *Puya* species and some

Pitcairnioideae species, and they found both *Puya* species nested within Pitcairnioideae (Krapp et al., 2014) which is highly incongruent with other published phylogenies (Givnish et al., 2007; Givnish et al., 2011; Schulte et al., 2013), including another PHYC phylogeny (Jabaily and Sytsma, 2010). *Puya* remained nested within Pitcairnioideae even when sampling was increased with additional *Puya* sequences from Jabaily and Sytsma (2010) that were taken from GenBank. Schütz et al. (2016) also found *Puya* nested within Pitcairnioideae in their PHYC phylogeny but not in their plastid phylogeny. Krapp et al. (2014) also warned about the use of PHYC as a molecular marker in Bromeliaceae. They found that PHYC had a high incidence of heterozygosity which can make PHYC trees a less accurate reflection of the species tree and can contribute to incongruent phylogenies between loci. These pieces of evidence cast doubt on the reliability of PHYC in Bromeliaceae and highlight the need for another phylogeny based on a different nuclear locus.

Hypothesis

Jabaily and Sytsma (2010) proposed a series of introgression and hybridization events to explain the chloroplast and nuclear incongruences between the Chilean taxa. First, they started with two separate lineages in Chile, a distinctive Blue *Puya* and Yellow *Puya,* a close relative of Core *Puya* (Fig. 1, step 1). In an ancient chloroplast capture event, a Blue *Puya* maternal plant crossed with a Yellow *Puya* pollen donor plant. The hybrid lineage did not persist and instead backcrossed into the paternal Yellow *Puya* population. In their offspring, over time the nuclear genome signal from Blue *Puya* became washed out, but the non-recombinant, maternally inherited Blue *Puya* chloroplast randomly became fixed in all subsequent Yellow *Puya* taxa (Fig. 1, step 2). As a result, no distinctive Yellow *Puya* chloroplast remains today. *P. chilensis* and *P. gilmartiniae* are the extant Yellow *Puya* taxa resulting from this chloroplast capture event. In the third step, a population, or a single individual of this post-chloroplast capture ancestor hybridized with Blue *Puya* to yield the ancestor of light-blue *P. alpestris,* a putative homoploid hybrid species. In the fourth step, *P. boliviensis* was created through a second chloroplast capture event between the Yellow *Puya* ancestor and a maternal Central Andean, Core *Puya* ancestor.

In 2013, Jabaily and Sytsma added to the body of knowledge about the *Puya* phylogeny with an

AFLP analysis (Jabaily and Sytsma, 2013). The AFLP technique is a broad scan of the nuclear genome, so it should yield a consensus nuclear topology. The AFLP phylogeny by Jabaily and Sytsma had some similarities and some differences to their 2010 PHYC phylogeny. It showed Blue *Puya* sister to Core *Puya,* and Yellow *Puya* embedded within Core *Puya,* rather than sister to Core *Puya.* This paper, with expanded taxon sampling, provided additional evidence supporting the strong conflict between the chloroplast phylogeny and the nuclear phylogeny specific to the *Puya* taxa from Chile, though it called into question the exact order of the steps in the hypothesis from Jabaily and Sytsma (2010). The AFLP technique is also falling out of favor for use in phylogenetics because it may be inaccurate. So, additional evidence from another nuclear locus is required to more fully support a hypothesis of Chilean *Puya* evolution. The region between the 8th and 10th exons of glyceraldehydes-3-phosphate-dehydrogenase (g3pdh), recently used in species-level bromeliad phylogenetic studies (Aguirre-Santoro et al., 2016; Sass & Specht, 2010), is used in this thesis as a second nuclear source to test Jabaily and Sytsma's (2010) hypothesis. The assumption is made that g3pdh and PHYC yield independent nuclear datasets because they are not members of the same gene family. Polyploidy is rare in Bromeliaceae and all *Puya* that have been surveyed are monoploid with N=25 (Smith and Downs, 1974; Brown and Gilmartin, 1989; Gitaí et al., 2005). Of 12 *Puya* species analyzed by Gitaí et al. (2014), none were polyploid, including the hybrid, *P. x berteroniana*. Viehmannova et al. (2016) even found that *P. x berteroniana* clonal pups were also monoploid. There is always a risk that a given nuclear gene has undergone lineage-specific duplications, however g3pdh was treated as a low-copy gene in Sass and Specht (2010) and Aguirre-Santoro et al. (2016), studies of Bromelioid genera, and there was no evidence of duplication noted. So the assumption is made here that g3pdh is monoploid and reliable for phylogenetic reconstruction. I will also use this g3pdh phylogeny in a molecular dating framework, which has not been done before, and I will hypothesize about the biogeographic history of the clade.

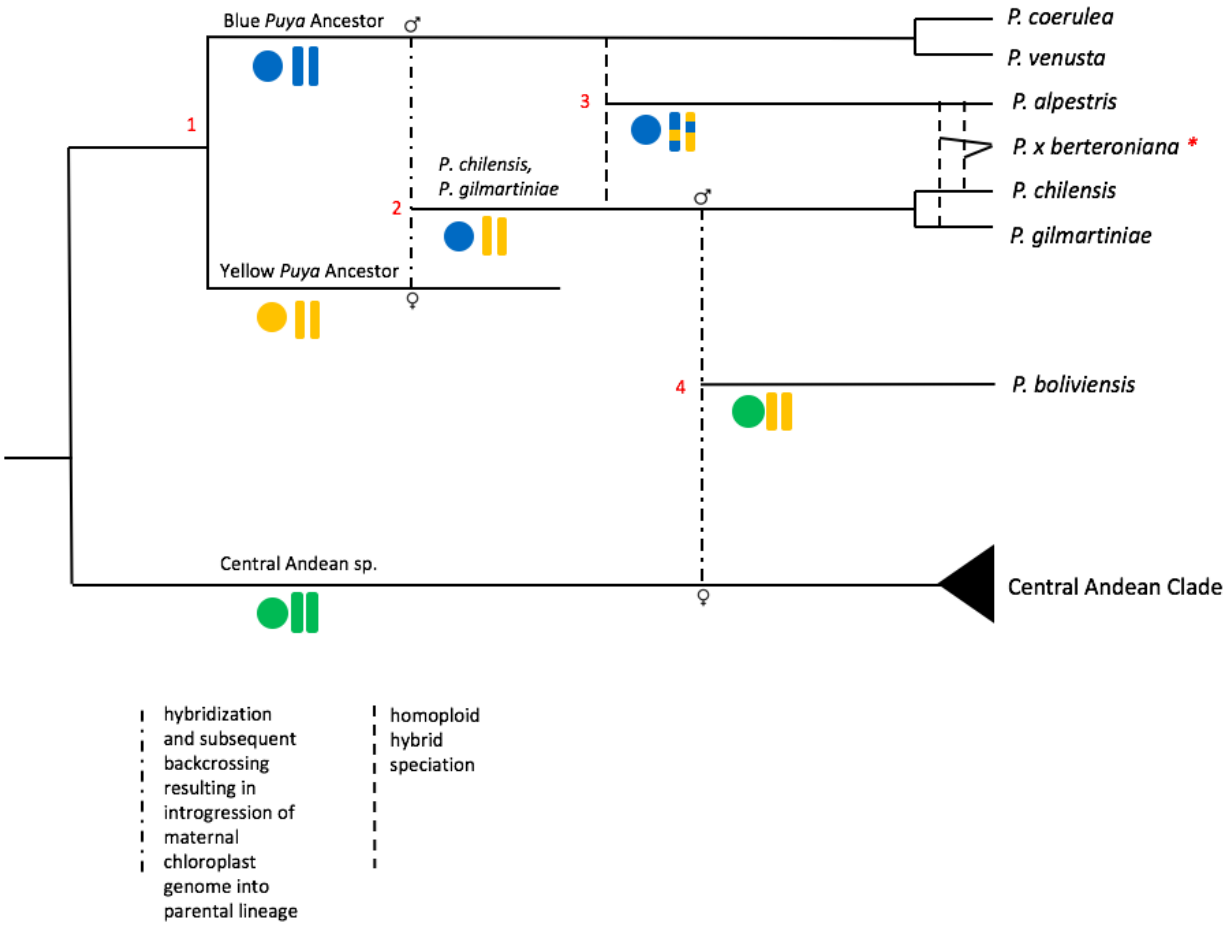

Figure 1. Hypothesis of molecular evolution in Chilean *Puya* species. Adapted from Jabaily and Sytsma, 2010 (Fig. 7) with additional hybridization inferred from Schulte et al., 2010 and Zizka et al., 2013, indicated with an asterisk. Events of interest are numbered in red. Circles represent the chloroplast genome. Bars represent the nuclear genome.

Molecular Dating Analysis

Phylogenies are often dated because, in the context of geological history, this provides insight into when and how specific lineage splitting events happened. Because branch lengths represent the amount of evolutionary change between lineages, if a few node ages can be estimated from direct fossil evidence, then the remaining node ages can be extrapolated. Givnish et al. (2011) published a time-calibrated phylogeny of the family Bromeliaceae. They found the stem age to be 100 million years ago (Ma) and the crown age to be about 19 Ma. This large gap between the stem and crown ages in this family suggests either that the family had low lineage formation until about 19 Ma, when a niche may have opened up or a key innovation propelled an increase

in speciation; or that much extinction occurred before the extant crown radiation. I will calibrate our g3pdh phylogeny to the inferred nodes from the analysis by Givnish et al. This will be the first molecular dating analysis focused on the genus *Puya*. The same dates will also be used to date the chloroplast phylogeny from Jabaily and Sytsma (2010), for the first time. Given the incongruences between Jabaily and Sytsma's nuclear and chloroplast phylogenies, it is reasonable to expect that dating of the chloroplast phylogeny may yield different results than dating of the g3pdh phylogeny. Both dated phylogenies will be used to reconstruct past events in the history of *Puya*.

Biogeography of Chile

The first biogeographic analysis of Chilean *Puya* was performed by Varadarajan in 1990. The analysis was based on field work and herbarium data and found that species are remarkably wide-ranging in their habitat type and geographic location with sympatry occurring often. Varadarajan proposed that *Puya* diverged in the Guayana Highlands, then moved to the northern Andes, followed by the central Andes, and from there into Chile (Varadarajan, 1990). In 2013, Jabaily and Sytsma performed a molecular biogeographic analysis of *Puya* and found that the genus originated in Chile instead. Givnish et al. (2011) corroborated that finding and also found that some early diverging lineages of Bromelioideae are from Chile as well.

Jabaily and Sytsma (2013) hypothesized that the diversification of *Puya* was driven by Andean uplift events. The first mountain-building events in South America occurred in the mid-Cretaceous in the southern tip of the continent, in the western parts of the Feugian and Patagonian Andes (Taylor, 1991). Uplift events continued periodically interspersed with times of stability. The general mountain-building trend was from west to east and south to north, not occurring in the northern regions (e.g. Ecuador, Colombia, Venezuela) until the Eocene, around 54.8 Ma. We are currently in middle of the sixth major uplift event, which started in the late Pliocene, around 3.6 Ma (Taylor, 1991). Many South American lineages have a history of allopatric speciation which follows Andean uplift events. Jabaily and Sytsma (2013) invoked (but did not directly test with molecular dating) the same history for *Puya*: as areas of land are uplifted, once contiguous populations are isolated from each other by altitudes that the plants

cannot survive at and thus promote allopatric speciation. In this thesis, I discuss the timing of lineage divergence in the context of biogeographical circumstances.

Previous phylogenies of *Puya* were limited by short loci with little variation. I resampled many of the taxa sampled by Jabaily and Sytsma (2010), this time sequencing g3pdh (Sass and Specht, 2010). G3pdh is longer and more phylogenetically variable. Thus, it is more informative than previous loci and serves as a third dataset for comparison with the PHYC and cpDNA topologies from Jabaily and Sytsma (2010). In addition to sampling the same extractions used in Jabaily and Sytsma 2010 and 2013, I re-extracted those with low DNA quantity. My new sampling includes all Chilean taxa and placeholder Northern and Central Andean taxa. I also apply this new phylogenetic analysis to the first-ever molecular dating analysis of *Puya.* Using these data, the previous hypothesis by Jabaily and Sytsma (2010), and a review of the literature, I update the working hypothesis for the history of Chilean *Puya*.

Materials and Methods

Taxon sampling

All except one accession are field collections made by R. Jabaily from 2005 to 2008, most of which are included in Jabaily and Sytsma, 2010 (Appendix A). One accession is from a collection made by collaborator Marcelo Rosas in Chile. Taxon sampling in this study was limited to the accessions already in the possession of R. Jabaily. All six recognized Chilean species were sampled. All accessions of the hybrid *P. x berteroniana* failed either PCR or sequencing. The remaining *Puya* taxa were selected because a Chilean taxon was potentially sister to it, based on the nuclear phylogeny from Jabaily & Sytsma (2010) or they serve as placeholders for the major groups within *Puya*. Those species are *P. assurgens, P. casmichensis, P. stenothyrsa, P. claudiae, P. dasylirioides, P. densiflora, P. floccosa, P. hamata, P. herrerae, P. macrura, P. maculata, P. mima, P. mirabilis, P. nana, P. nitida, P. novarae, P. nutans, P. obconica, P. pearcei, P. raimondii, P. tillii, P. trianae, P. weberbaueri, P. wrightii,* and *P. yakespala.* Eight outgroup taxa are included which represent seven genera from the sister subfamily Bromelioideae.

Givnish et al. (2011) identified three groups within Bromelioideae based on morphology and geography and I sampled within each of those clades. They identified the "Brazilian Shield" clade, "Tank Epiphytes", and those taxa that are in neither clade. From the core Bromelioideae, which is inside the tank epiphyte group, which is inside the Brazilian Shield clade, I sampled *Aechmea magdalenae, Lymania spiculata,* and *Araeococcus pectinatus.* Outside of core Bromelioideae but still inside of the tank epiphyte group I sampled *Ronnbergia deleoni*. Outside of the Tank Epiphyte group, but still inside of the Brazilian Shield clade, I sampled *Ananas comosus* and *Acanthostachys strobilacea.* The two Bromelioids I sampled outside of these groups were *Bromelia flemingii* and *Bromelia trianae*. G3pdh sequences for the outgroups were taken from GenBank accessions by Sass and Specht, 2010 and Aguirre-Santorro et al., 2016.

DNA extraction, amplification, and sequencing

Total DNA was extracted from silica-dried leaf tissue. Initial extractions were made between 2006 to 2008 by RSJ, and some samples were re-extracted from excess tissue or extracted for the first time in 2019 by me. Extractions made between 2006 and 2008 used the DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA). Extractions made in 2019 followed the DNeasy Plant Pro Kit (Qiagen, Valencia, California, USA) with the following modifications. Bead-beating was replaced with by-hand mortar and pestle maceration. Two to five times the recommended amount of solution CD1 was used in the first step because I found that it yielded higher DNA concentrations. DNA quantity was evaluated using a Qubit 4.0 fluorometer (Thermo Fisher Scientific, Wilmington, Delaware, USA).

G3pdh primer pairs (forward: 5'-CAT CTA GCA AGG ACT GGA GAG G-3') (reverse: 5'-GCT GAA GAT ACC TGC TGT CAC C -3') followed Sass and Specht (2010). Our timeline did not permit us to design more specific primers for *Puya*, and these primers generally yielded strong single bands for most taxa.

Polymerase chain reaction (PCR) was carried out in 25 µl reactions in the first round and in 50 µl reactions in the second round in an effort to increase yield. Reaction mixtures of 25 µl total

volume were comprised of 12.5 µl of GoTaq Green mixture (Promega, Madison, Wisconsin, USA), 1.25 µl of dimethyl sulfoxide (DMSO), 1 µl of 20 µM forward primer, 1 µl of 20 µM reverse primer, 8.25 µl of ddH2O with 1 µl of DNA template. 50ul reaction mixtures were comprised of 25 µl of GoTaq Green mixture, 2.5 µl of DMSO, 2 µl of 20 µM forward primer, 2 µl of 20 µM reverse primer, 16.5 µl of ddH2O, and 2 µl of DNA template. Thermal cycling was carried out in a MultiGene OptiMax Thermal Cycler (Labnet International, Edison, New Jersey, USA). Thermal cycling conditions followed Aguirre-Santoro et al., 2016: 1 cycle of 3 min at 95°C, followed by 8 cycles of 45 s at 95°C, 45 s at 59°C, and 45 s at 72°C, followed by 27 cycles of 1 min at 95°C, 1 min at 65°C, 1.5 min at 72°C, followed by 1 cycle of 7 min at 72°C.

Amplification of the locus ETS was attempted with multiple rounds of trouble-shooting. PCR was performed following the protocol by Aguirre-Santoro et al. (2016) with primers from Sass and Specht (2010). There were problems with double bands so the Sass and Specht touchdown protocol (2010) was used. Again, there were double bands indicating imprecise primers for our taxa, a problem not noted in Bromelioideae. Gel extraction of the larger bands following the Qiagen QIAquick Gel Extraction Protocol did not yield high enough product volume and subsequent sequences were unusable.

The g3pdh PCR product was bead-cleaned using HighPrep PCR beads (MagBio Genomics, Gaithersburg, Maryland, USA) following the manufacturer's protocol. All DNA was eluted into 40ul of deionized, distilled water. Samples were Sanger sequenced at Macrogen (Seoul, South Korea)

Sequence alignment and phylogenetic analysis

Forward and reverse sequences for each successfully amplified extraction were assembled into single final sequences using the "High Sensitivity / Medium" setting in Geneious (Geneious Prime 2019.1.3 (https://www.geneious.com) Kearse et al., 2012). The ends were trimmed and ambiguity codes were checked and added manually. Ambiguity was visualized as multiple different nucleotide peaks at the same base pair position. This could be the result of multiple PCR products, but it is more likely a sign of heterozygosity because ambiguities occurred in

isolated places and not often. Gaps across all taxa were stripped. No indels existed because g3pdh is a coding region. The 35 *Puya* accessions ranged from 170 bp in length (*P. coerulea*) to 1,008 bp (*P. mima*), resulting in a total alignment length of 1,187 bp.

RAxML v.8 (Stamatakis, 2014) in Geneious was used to generate a Maximum Likelihood phylogeny. To determine the appropriate Bayesian Information Criterion (BIC), jModelTest v. 2.1.10 (Darriba et al., 2012) was used. GTR was used in RAxML because it was the closest available model in the program to the jModelTest suggestion. One hundred bootstrap replicates were run. The consensus tree was made with a support threshold of 70%. The resulting tree is shown in Figure 2.

MrBayes v. 3.2.6 (Ronquist et al., 2012) in Geneious was used to run a Bayesian Posterior Probability analysis. GTR was used as the model again. Gamma rate variation was specified. The first 25% of trees were discarded as 'burn-in.' The resulting tree is shown in Figure 3.

Hypothesis Testing

Various alternative hypotheses were written as constraints and tested against the data to determine if the resultant trees were significantly worse fits to the data than unconstrained analysis. Templeton tests of each hypothesis were conducted in PAUP 4.0 beta, following Jabaily and Sytsma, 2010.

Hypothesis tests were run on a different alignment that included only outgroups *Aechmea magdalenae, Ananas comosus,* and *Ronnbergia deleoni,* and did not include *P. coerulea*. All other *Puya* sequences were exactly the same as the ones used in the alignments for the ML, BI, and molecular dated trees.

Molecular Dating

Molecular dating was carried out using the program BEAST v1.10.4 (Drummond et al., 2006). jModelTest again showed that GTR was the most appropriate available model of nucleotide

evolution. Default settings were used in all cases except for the following. The clock type was set to uncorrelated relaxed clock and the tree prior was set to speciation yule process. Default priors were used except that the time to most recent common ancestor (tmrca) for all taxon sets was changed to normal with a standard deviation of 1.0 Ma. Chain length was 100,000,000.

There are no known fossils within Bromeliaceae so secondary calibration points were used from Givnish et al. (2011), which has a molecular dated phylogeny with eight *Puya* species included. I was not able to obtain g3pdh sequences for Pitcairnioideae, the subfamily sister to Bromelioideae plus Puyoideae, which would have served as an outgroup and a point of time calibration for the split between Puyoideae and Bromelioideae. Instead, I sampled within Bromelioideae in order to use this second time calibration point outside of *Puya*. Three taxon sets were defined following Givnish et al. (2011). *Puya* was constrained to be monophyletic. Within Bromelioideae, Tank Epiphytes and the Brazilian Shield clade were also constrained to be monophyletic. The following calibration points were coded. The split between Puyoideae and Bromelioideae was 10.1 Ma (Givnish et al., 2011). The split between the Brazilian Shield clade and the rest of Bromelioideae was 9.1 Ma (Givnish et al., 2011). The split between Tank Epiphytes and the rest of the Brazilian Shield clade was 5.5 Ma (Givnish et al., 2011). All calibration points used normally distributed priors with a standard deviation of 1.0 Ma.

In order to compare potential differences in dates between chloroplast and nuclear loci, I used the *matK, trnS-trnG,* and *rps16* chloroplast sequences from Jabaily and Sytsma (2010), which I obtained from GenBank, and ran a BEAST analysis on the concatenated alignment. The same methods were used to date this phylogeny.

Results

The g3pdh dataset is more phylogenetically informative than the previous datasets from Jabaily and Sytsma (2010). It is longer than the individual cpDNA alignments and slightly longer than the PHYC alignment (Table 1). It is far more parsimony informative than the cpDNA loci and slightly more parsimony informative than PHYC.

Dataset	Total Characters	Variable and Parsimony Uninformative	Parsimony Informative Characters	Percent Parsimony Informative
g3pdh	1178	105	113	9.59%
PHYC	1048	178	97	9.3%
matK	819	77	32	3.9%
trnS-trnG	1048	104	49	5%
rps16	827	108	43	5.2%

Table 1. Dataset statistics for g3pdh, PHYC, *matK, trnS-trnG,* and *rps16* datasets. The g3pdh alignment included only outgroups *Aechmea magdalenae, Ananas comosus,* and *Ronnbergia deleoni* and did not include *P. coerulea*. The information for remaining loci used alignments that included outgroups and was found in Jabaily and Sytsma (2010), Table 3.

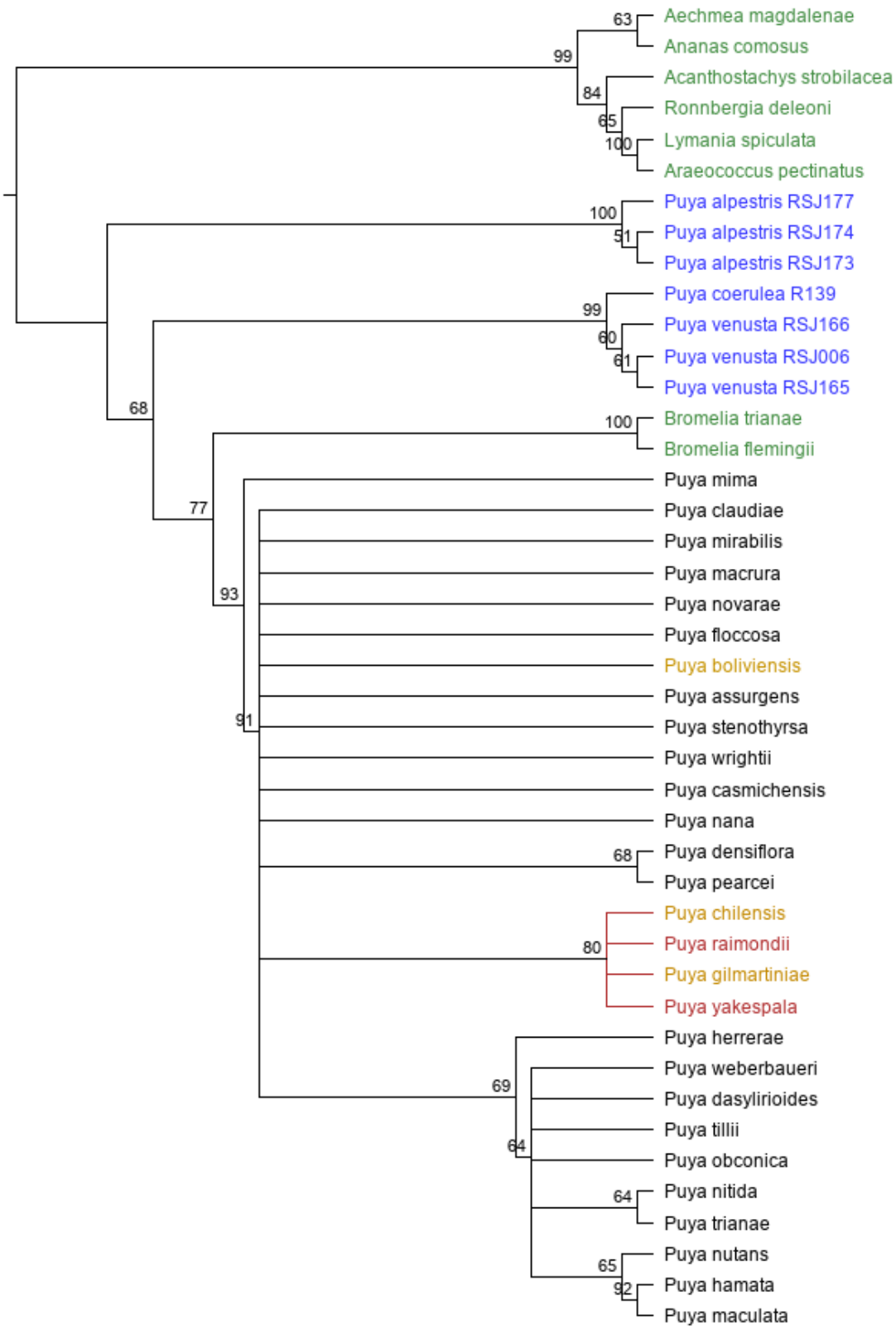

Figure 2. Maximum Likelihood phylogeny with bootstrap support of Chilean *Puya* with other representative *Puya* and Bromelioideae outgroups. Outgroups are green. Blue *Puya* taxa are blue. Yellow *Puya* taxa are yellow. The Elevational Disjunct clade (discussed later) is red.

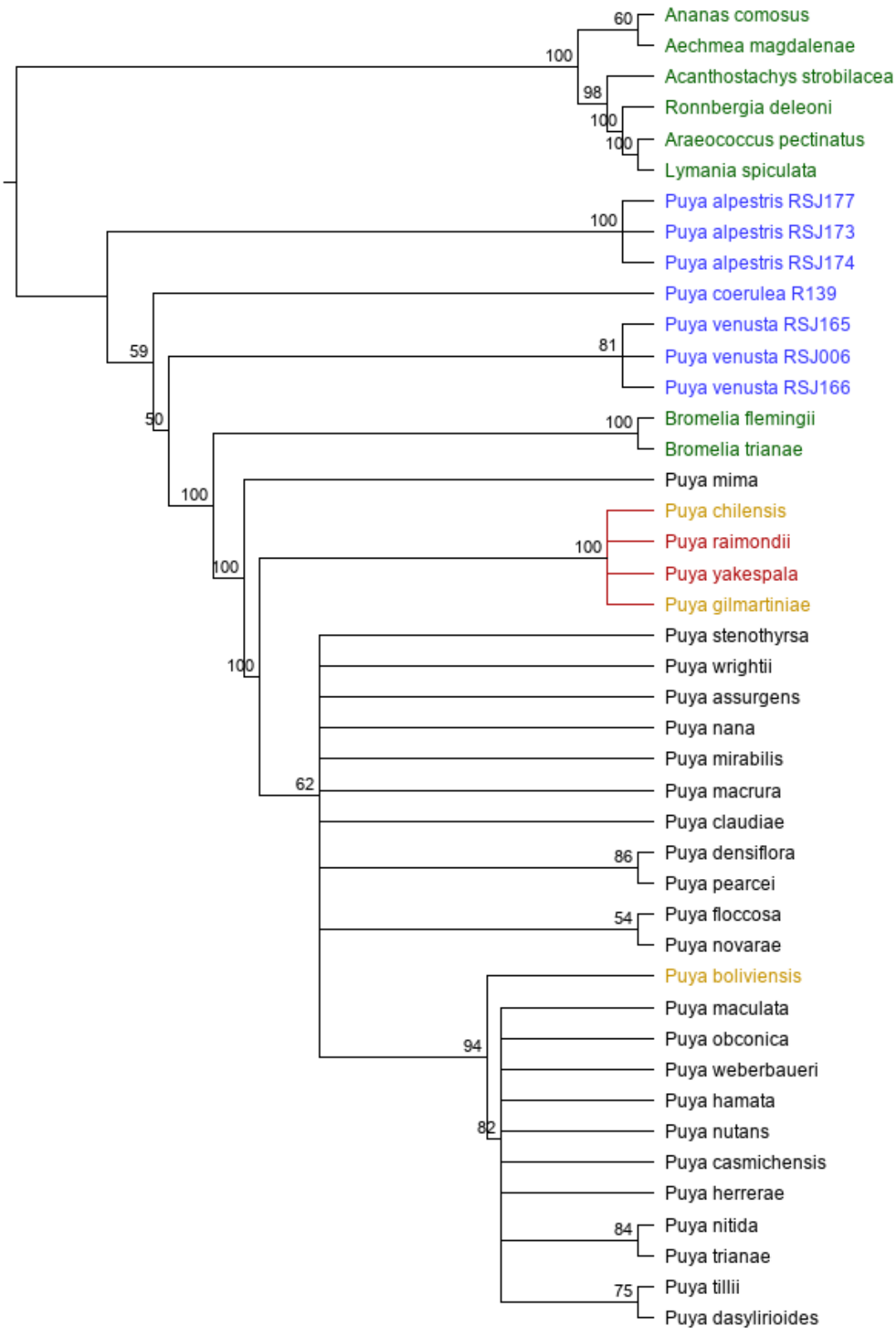

Figure 3. Bayesian Inference phylogeny of Chilean *Puya* with other representative *Puya* and Bromelioideae outgroups. Outgroups are green. Blue *Puya* taxa are blue. Yellow *Puya* taxa are yellow. The Elevational Disjunct clade is red.

Maximum Likelihood and Bayesian Inference Phylogenies

The backbone of the Maximum Likelihood Bootstrap support (ML) phylogeny places *Aechmea, Ananas, Acanthostachys, Ronnbergia, Lymania,* and *Araeococcus* in the outgroup position with bootstrap support of 99 for that clade (Figure 2). The other Bromelioideae species, *Bromelia trianae* and *Bromelia flemingii* are placed within *Puya,* sister to Core *Puya*, making Bromelioideae paraphyletic, although the backbone nodes supporting this placement have low support. There is high support for the monophyly of all *Puya* plus *Bromelia*. There is low support for *P. alpestris* as sister the remainder of that clade, and there is low support for the next clade of *P. coerulea* and *P. venusta* as stepwise sister to the rest of *Puya* and *Bromelia,* inside of *P. alpestris*. All accessions from the same species did group monophyletically. The two species of *Bromelia* are sister to Core *Puya,* a well-supported clade. *Puya mima* is sister to the remainder of Core *Puya,* also with strong support. Within Core *Puya* the Chilean taxon *P. boliviensis* is embedded in a well-supported polytomy with Core *Puya*. In the same polytomy, the remaining Chilean *Puya*, *P. chilensis* and *P. gilmartiniae* form a clade with *P. raimondii* and *P. yakespala.* This clade is supported with bootstrap support of 80, and is a novel finding. Structure is only resolved within Core *Puya* in two other poorly supported clades.

More structure was resolved with the Bayesian Inference posterior probability (BI) phylogeny (Figure 3). *Aechmea, Ananas, Acanthostachys, Ronnbergia, Lymania,* and *Araeococcus* again form the outgroup. This relationship has a posterior probability of 100. The two species of *Bromelia* are again embedded within *Puya* and this broader clade receives a posterior probability of 100. Like the ML phylogeny, the two nodes placing *Bromelia* within *Puya* receive very low support. *Puya alpestris* alone falls to the base of *Puya* plus *Bromelia*. Unlike the ML phylogeny, *P. coerulea* falls sister to the rest of *Puya* plus *Bromelia* apart from *P. venusta* but this relationship has low support. The *P. alpestris, P. coerulea, P. venusta* relationship is minimally supported, with low posterior probability and short branch lengths. Again, all accessions of the same species were monophyletic. The posterior probability for the position of *Bromelia* sister to Core *Puya* is 100. *P. mima* again falls to the base of Core *Puya* with a posterior probability of 100. With *P. mima*, the BI analysis provides important structure where the ML analysis did not. *P. chilensis, P. gilmartiniae, P. raimondii,* and *P. yakespala* again form an unstructured clade,

and that clade is placed at the base of the remaining Core *Puya.* There is some structure among Core *Puya* which is similar in many respects to the ML phylogeny.

Hypothesis Testing

A monophyletic Chilean group was rejected (P<0.0001). A monophyletic Chilean group minus *P. boliviensis*, essentially replicating the chloroplast hypothesis of Jabaily and Sytsma (2010), was rejected (P<0.0001). A monophyletic Blue *Puya* clade (*P. alpestris* and *P. venusta*) was not rejected (P=0.2972). This is consistent with the low support found for its paraphyly. A monophyletic Yellow *Puya* clade (*P. chilensis, P. gilmartiniae,* and *P. boliviensis*) was rejected (P=0.0246). A monophyletic Core *Puya*, splitting up the Elevational Disjunct clade, was rejected (P=0.0083). Finally, a monophyletic subgenus *Puya* was rejected (P=0.0001).

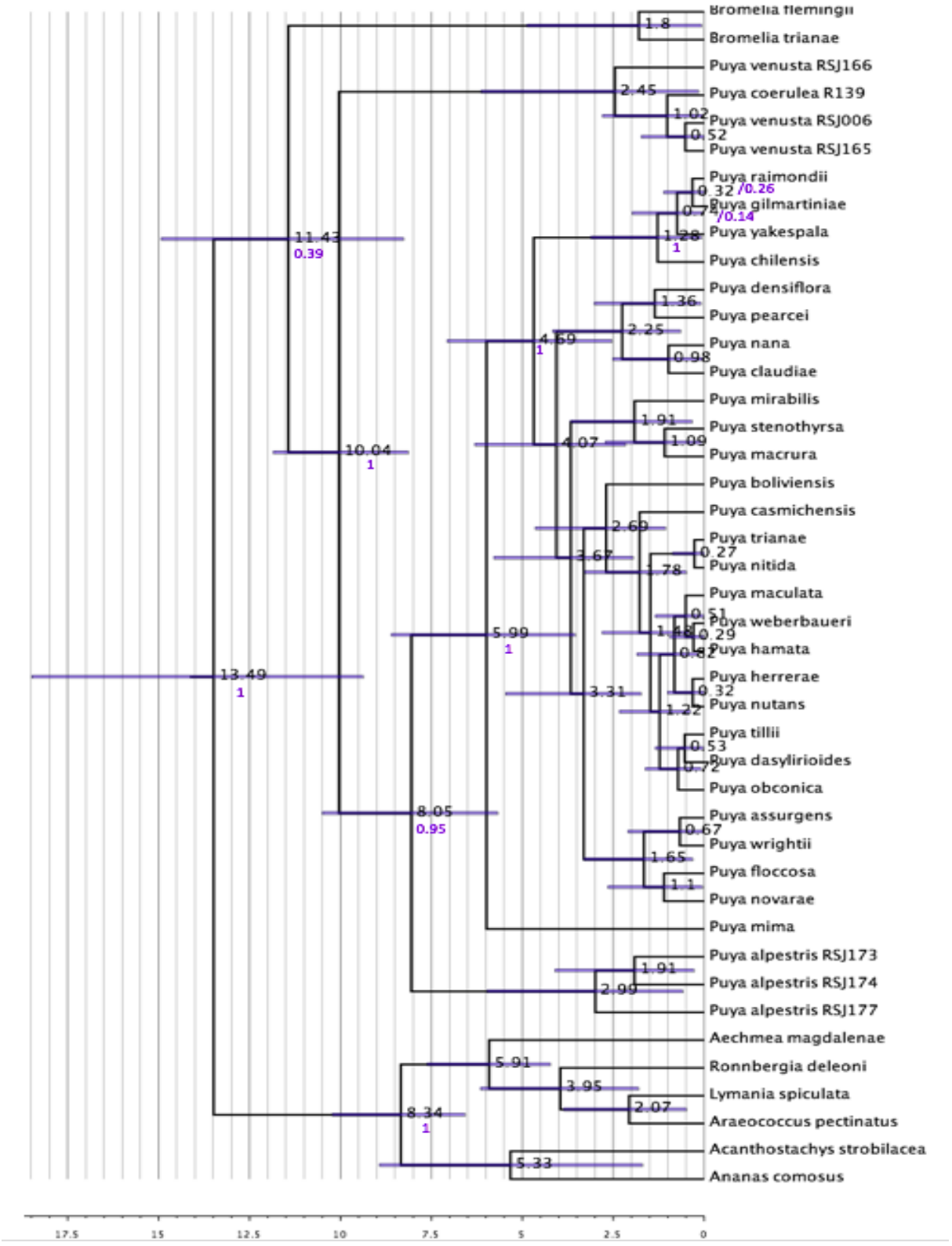

Figure 4. Molecular dated phylogeny with Bayesian Inference. Values in black are median age estimates in millions of years. Values in purple are posterior probability shown at important nodes. Secondary calibration points were placed in outgroup taxa, between the two *Bromelia* species, and between *Aechmea, Ronnbergia, Lymania, and Araeococcus*, and the rest of Bromelioideae. See Appendix B for all posterior probabilities.

Molecular Dating and Biogeography

A molecular dating analysis was run on the alignment using a Bayesian Inference framework. This analysis dated lineage splitting events and those ages are used in the context of geological events in South America to gain insight into the formation of the genus *Puya.* The mean node age for the split between *Puya* and *Bromelia* is 11.4 Ma (Figure 4), which is close to what Givnish et al., 2011 found (10.1 Ma). *Puya venusta* plus *P. coerulea* and *P. alpestris* are the earliest diverging lineages of the *Puya* sampled, but they are not sister. *Puya coerulea* is embedded within *P. venusta. P. venusta* and *P. coerulea* diverge first, at 10.04 Ma and *P. alpestris* diverges next, at 8.05 Ma. The divergence of *P. venusta* before *P. alpestris* is incongruent with both the ML and BI phylogenies, where *P. alpestris* diverged first but the posterior probability for this relationship is 1. Just like the ML and BI phylogenies, *P. mima* falls sister to the rest of Core *Puya*, diverging at 5.99 Ma. Again, a *P. chilensis, P. gilmartiniae, P. raimondii,* and *P. yakespala* clade is resolved. The structure of that clade shows *P. chilensis* sister to the other two, diverging at 1.28 Ma. *P. yakespala* diverges next, at 0.74 Ma, and *P. raimondii* and *P. gilmartiniae* split at 0.32 Ma. The posterior probability for *P. chilensis* sister to the other three taxa is 1. The other two relationships have low support.

Figure 5. Molecular dated phylogeny of *matK, trnS-trnG,* and *rps16* chloroplast regions from Jabaily and Sytsma, 2010. Posterior probability shown at important nodes in black below or after node date. A secondary calibration point was used at the split between *Puya* and *Ananas comosus*. See Appendix C for all posterior probabilities.

Chloroplast Dated Phylogeny

The concatenated cpDNA dataset from Jabaily and Sytsma (2010) was dated for the first time (Figure 5) and, as expected, it yielded a very different topography from g3pdh. Sampling was slightly different but taxa of note were clearly related differently in the tree. This analysis found the date of the split between Bromelioideae and Puyoideae to be 10.96 Ma, very close to the findings of the cpDNA phylogeny from Givnish et al. (2011), and about half a million years later than the date from the g3pdh analysis. Chilean *Puya,* excluding *P. boliviensis,* split from Core *Puya* 9.81 Ma. After that there was a relatively long period where no extant lineages split off and northern/central Andean Core *Puya* and Chilean *Puya* accumulated much molecular difference. 5.81 Ma Core *Puya* began to split and, at least with this sampling, continued to split regularly until the present. Extant lineages of Chilean *Puya* did not begin to diversify until 2.86 Ma.

Discussion

In 2010, Jabaily and Sytsma found incongruences between a PHYC and a chloroplast phylogeny of *Puya*. That prompted them to hypothesize a history of chloroplast capture and hybridization in the lineage. In this study, I tested that hypothesis with a new nuclear locus, g3pdh. G3pdh corroborated the PHYC topology by showing a non-monophyletic Chilean *Puya*. But it also added novel evidence suggesting two distinct Blue *Puya* groups, the *P. alpestris* group, and the *P. coerulea* and *P. venusta* group. G3pdh also placed Yellow *Puya* in a novel position, embedded in Core *Puya* with some surprising taxa from high elevation habitats. These findings are incorporated into the existing knowledge and below I update the hypothesis by Jabaily and Sytsma (2010).

G3pdh topology compared to cpDNA and PHYC

G3pdh is a nuclear locus, and our results partially corroborate those of Jabaily and Sytsma's (2010) PHYC nuclear phylogeny. In that phylogeny, Chilean *Puya* was paraphyletic, with Blue *Puya* sister to Yellow *Puya* plus Core *Puya* and Yellow *Puya* sister to Core *Puya*. Blue and

Yellow *Puya* were, however monophyletic within themselves. Both our ML and BI phylogenies show a paraphyletic Blue *Puya* sister to Yellow *Puya* plus Core *Puya*, with members of Yellow *Puya* embedded within Core *Puya* in non-monophyly. This is the first evidence that Blue *Puya* may not be monophyletic. However, hypothesis testing failed to reject a monophyletic Blue *Puya* clade including, *P. alpestris* and *P. venusta* (P=0.2972), and I obtained low support values for the paraphyly of these species. So, this evidence is preliminary. In contrast, a monophyletic Yellow *Puya* clade, including *P. chilensis, P. gilmartiniae,* and *P. boliviensis,* was rejected (P=0.0246), supporting our finding that Yellow *Puya* has a closer history with Core *Puya* than previously thought.

As expected, results do not corroborate Jabaily and Sytsma's 2010 chloroplast phylogeny. Their chloroplast phylogeny showed a monophyletic Chilean *Puya* (except for *P. boliviensis,* which was embedded within Core *Puya*) in a polytomy with Core *Puya* and Bromelioideae, whereas the g3pdh phylogeny yields non-monophyly of Chilean *Puya.* Hypothesis testing solidified this conclusion by rejecting a monophyletic Chilean group (P<0.0001). Not only does the g3pdh locus yield non-monophyly of Chilean *Puya*, but it shows Yellow *Puya* taxa in a more derived position. Yellow *Puya* occupied a basal position in the PHYC phylogeny, sister to Core *Puya*, but it was embedded in Core *Puya* in the g3pdh phylogeny. Although there is low support for the paraphyly of Blue *Puya* and its basal position, there is high support for Yellow *Puya* embedded within Core *Puya.* While these incongruences add further uncertainty to the evolutionary process of *Puya*, they do corroborate the close relationship between Yellow *Puya* and Core *Puya* shown in the PHYC phylogeny; and the basal position of Blue *Puya* in the genus.

In their hypothesis, re-interpreted in Fig. 1, Jabaily and Sytsma (2010) say that *P. coerulea* and *P. venusta* should be closely related because they are the descendants of the Blue *Puya* lineage and they show no evidence of past chloroplast capture or hybridization with other lineages. Our results support that hypothesis, the monophyly of *true* Blue *Puya*. The ML phylogeny shows *P. coerulea* sister to *P. venusta* with bootstrap support of 99%. The BI phylogeny places *P. coerulea* to the base of the *P. venusta* plus sister groups clade, instead of sister to *P. venusta*. However, I suspect that that is an artifact of the short sequence I obtained for *P. coerulea* (170 bp, whereas the alignment was 1,187 bp), rather than a true representation of the species

relationship, because it is poorly supported. The Bayesian framework used in the BEAST analysis for molecular dating also breaks up the *P. coerulea* / *P. venusta* clade. I attribute that to the short sequence as well, because it was also poorly supported.

According to the hypothesis by Jabaily and Sytsma (2010), *P. alpestris* is a homoploid hybrid between Blue *Puya* and the *P. chilensis, P. gilmartiniae* Yellow *Puya* ancestor; and *P. x berteroniana* is a homoploid hybrid between *P. alpestris* and the *P. chilensis, P. gilmartiniae* ancestor. Jabaily and Sytsma suspect that they are hybrids because of their intermediate morphology between Yellow and Blue *Puya,* and its incongruent placement in the chloroplast versus nuclear-derived phylogenies. Schulte et al. (2010) used AFLP analysis to create a phylogeny of Chilean *Puya* with many accessions per species and found evidence for hybridization. They found three groups, the "alpestris" group, comprising all accessions of *P. alpestris* and *P. berteroniana*, the "chilensis" group comprising all accessions of *P. chilensis, P. gilmartiniae,* and *P. boliviensis,* where the three species were monophyletic, and the "coerulea" group, comprising all accessions of *P. venusta* and *P. coerulea,* where the two species were monophyletic. Varieties of *P. coerulea* were not all monophyletic. They showed that *P. alpestris* and *P. berteroniana* were not monophyletic in the *P. alpestris* - *P. berteroniana* species complex, suggesting that hybridization occurs among extant populations. They also found evidence of hybridization in sympatric populations of the alpestris group and the chilensis group. Their STRUCTURE analysis did not find that either *P. alpestris* or *P. berteroniana* were recent hybrids although this could be because too much time has passed since the hybridization event. In 2013, Zizka et al. renamed *Puya berteroniana* "*Puya x berteroniana"*. Their herbarium work revealed that the name *P. berteroniana* had, in some cases, been misapplied to the northern population of *P. alpestris* because of their very similar morphologies. They use evidence from Schulte et al. (2010) and their own herbarium and field work to assert that *P. berteroniana* is a homoploid hybrid, and not a full species.

Our results do not refute a hybrid origin of *P. alpestris,* but beyond that it is difficult to interpret the meaning of the position of *P. alpestris* in our phylogenies. If it was a recent hybrid between Blue *Puya* and the *P. chilensis, P. gilmartiniae* ancestor, I would expect to see it group with Blue *Puya* based on some loci, and group with the *P. chilensis / P. gilmartiniae* ancestor based on

other loci. If it was a hybrid parental species for *P. x berteroniana,* I would expect to see it group with *P. x berteroniana* at some loci as well. The BEAST analysis found *P. alpestris* to be most closely related to Yellow and Core *Puya*, with *P. venusta* sister to all (posterior probability of 0.95). Our ML and BI reconstructions place *P. alpestris* at the base of *Puya,* equally related to *P. venusta*, Yellow *Puya,* and Core *Puya*. The PHYC phylogeny places *P. alpestris* in the monophyletic Blue *Puya* clade (Jabaily and Sytsma, 2010). The incongruences between reconstructions both based on nuclear DNA could be interpreted to indicate that different regions of the nuclear genome track different gene histories and that *P. alpestris* is a hybrid species. However, that conclusion is not certain because *P. alpestris* does not group with either putative parent lineage in our analyses. Interestingly, the monophyly of *P. alpestris,* as a species, is a novel finding of this paper, and it is highly supported in all analyses. I was unable to sequence *P. x berteroniana* to check whether it is, in fact, closely related to *P. alpestris*.

Our analysis found Bromelioideae to be non-monophyletic. However, when it was constrained to be monophyletic for the molecular dating analysis, that relationship was highly supported (posterior probability of 1). While it is unlikely that *Bromelia* is truly sister to Core *Puya*, inside Blue *Puya,* because of the body of work showing Puyoideae sister to Bromelioideae (Schulte et al., 2009; Jabaily and Sytsma, 2010; Givnish et al., 2011; Givnish et al., 2013), our findings do illustrate the complexity of the relationships in the backbone. Intergeneric crosses are known to occur in Bromelioideae (Benzing, 1980) which may explain the unresolved backbone of Bromelioideae and Puyoideae.

Molecular dating analysis and discussion of biogeography

The Chilean species of *Puya* occur almost exclusively at lower elevations in Chile. They generally occur below 1000 m, except for *P. alpestris* and *P. coerulea*, which occur up to 2200 m (Zizka et al., 2013). Chile is at the highest latitude of *Puya*'s range so its preference for low elevations may be a response to the greater seasonality at higher latitudes which compounds the stresses of living at high elevation. At low elevations in Chile, there are two dominant ecological regions. In the north is the Atacama Desert, the driest desert in the world. There, vegetation only exists in "Lomas," discrete areas of higher water availability. Outside of the Lomas there is no

vegetation (Dillon & Hoffmann, 1997). One species of *Puya* exists in, and is endemic to, Atacama, *P. boliviensis.* Much of the low-elevation area in Chile that is not the Atacama Desert is known as the Chilean Winter Rainfall - Valdivian Forest and is a hotspot of biodiversity. The Chilean Winter Rainfall-Valdivian Forest runs from about 30°S to 39°S (Arroyo, 1995) and contains almost 4,000 vascular plant species, about half of which are endemic (Critical Ecosystem Partnership Fund, n.d.). This region has a Mediterranean-type climate (MTC). There are only five MTCs globally and they all occur on the southwest edges of continents as a result of global air circulation (Joffre & Rambal, 2001). In 1990, Köppen defined MTCs as areas of mid-latitude that primarily receive rain in the winter, resulting in cool, wet winters and hot, dry summers.

Chile's MTC varies slightly from north to south. The north is xerophytic matorral and savannas. The central region has broad-leaved and sclerophyllous forests which turn into deciduous forests in the south (Arroyo, 1995). Most vegetative growth happens in the early spring when temperatures have risen but there is still moisture. The hot, dry summers are characterized by a fire regime which is an important selective pressure for organisms evolving in the region. The genus *Puya* is generally well-adapted to aridity, which is congruent with evolution in an MTC.

Node ages from our molecular dating analysis generally match those found by Givnish et al., 2011, the paper from which secondary calibration points were taken. Our results show the split between Puyoideae and Bromelioideae at 11.4 Ma. The onset of the Mediterranean-type climate was only about 3.2 Ma according to Raven in Di Castri and Mooney, 1973, and during late Miocene or early Pliocene (around 5.3 Ma) according to Thrower and Bradbury, 1973. This precludes the possibility that the MTC was the cause of diversification within Bromeliaceae leading to the splitting of the *Puya* lineage. The beginning of seasonality in what are now MTC climates, as a result of mountain uplift events and other global shifts, occurred closer to the Puyoideae / Bromelioideae split, at 12-15 Ma (Rundel et al., 2016). The start of the latest Andean uplift (3.6 Ma) is later than our findings for the origin of *Puya* (10.04 Ma). There is debate about exactly when aridification of the Atacama Desert began, but the range of possible dates (Pliocene to Miocene) (Ritter et al., 2018) leaves open the potential that the Atacama drove diversification of Bromeliaceae. The onset of the MTC and the start of the latest Andean uplift cannot explain

crown radiations of *Puya*, however these geographic events do line up with many of the most recent diversification events which cluster around 1.5 to 2 Ma with this sampling. A far greater sampling and larger dataset would be necessary to draw further conclusions about divergence times at the species level. Almost all of these most recent events are poorly supported (Appendix B).

Not only are the oldest lineages of *Puya* from Chile, so are the oldest lineages of Bromelioideae. Givnish et al. (2011) found that early diverging lineages in Bromelioideae are endemic to Chile, supporting the hypothesis that Chile is a major location of cladogenesis for all Bromeliaceae. Givnish et al. (2011) also found that support for the subfamilies Puyoideae and Bromelioideae increased when Chilean *Puya* and Chilean Bromelioids were removed from the phylogeny. This suggests that whatever evolutionary process caused the incongruence observed in *Puya* may also have affected Bromelioideae. Andean uplift is thought to have caused diversification in many lineages spanning the length of the Andes (Antonelli et al., 2009). But as a center of diversification in Bromeliaceae, it remains unknown what is unique about Chile. Some lineages in Mediterranean Chile are shared among Gondwanan land-forms, but most lineages evolved from temperate South American species (Joffre & Rambal, 2001).

The non-monophyly of Blue *Puya* led to incongruences with the dated phylogeny by Givnish et al. (2011). Givnish et al. found the crown radiations for Andean and Chilean *Puya* groups to be 3.5 Ma and 2.5 Ma respectively, around the beginning of the last Andean uplift event. The split between Andean and Chilean *Puya* was found to occur around 10 Ma, immediately after the split of Puyoideae from Bromelioideae. The branch lengths between that split and the crown radiations in these groups are, therefore, long. This finding is fascinating but was not corroborated with our data. I found the date of the split between Puyoideae and Bromelioideae to be close to 11.43 Ma from Givnish. However, only the *P. venusta* / *P. coerulea* clade split off then and another 2 million years passed before *P. alpestris* split. The remaining Core *Puya* lineage-splitting events that occurred after that filled the space evenly, with minimal long branches. The crown age of Andean *Puya* was 5.99 Ma, before the beginning of the last Andean uplift event.

Givnish et al. (2011) found that the Brazilian Shield clade split from the rest of Bromelioideae around 9.1 Ma and I found that split at 13.49 Ma. They found that the Tank Epiphyte clade split from the rest of the Brazilian Shield clade around 5.5 Ma and I found that it split around 5.9 Ma. Thus, there is no trend to the differences between our findings and those of Givnish et al. (2011).

Givnish et al. (2011) used a chloroplast phylogeny for their molecular time calibration, so the differences between chloroplast and nuclear phylogenies should be taken into account when considering the accuracy of our dates. However, the time scale I am dealing with is small enough that our dates should be close. In addition, our sampling within Puyoideae and Bromelioideae is incomplete, but Elizabeth Spriggs and colleagues show that the node dates generated should be accurate even when sampling is small (Spriggs et al., 2015).

In 2018, Givnish et al. published the most extensively-sampled monocot-wide phylogeny. It was reconstructed using the 77 plastid genes, so there were more data supporting it than the phylogeny from 2011, but sampling was less extensive within Bromeliaceae so that date was not used in this analysis. It sampled only one *Puya* species, *P. laxa*, two Bromelioideae species, both from the genus *Neoregelia*, and six other Bromeliads and showed the age of the split between Bromelioideae and Puyoideae to be more recent, at 7.8 Ma (Givnish et al., 2018, Appendix S13).

The Elevational Disjunct clade and an updated hypothesis

A new and interesting clade was reconstructed with high support in all three analyses. It comprises two Chilean species, and two Core *Puya* species: *P. chilensis, P. gilmartiniae, P. raimondii,* and *P. yakespala.* I refer to this as the Elevational Disjunct clade (ED clade). As discussed above, *P. chilensis* has shorter, broader, bright yellow to yellow-greenish flowers and its leaves are glabrous on the abaxial surface. *Puya gilmartiniae* has the same flowers but its leaves have a cinereous indumentum (Schulte et al., 2010). These two Chilean species plus *Puya raimondii* belong to the subgenus *Puya* with sterile inflorescence tips. *Puya raimondii* and *P. yakespala* had not previously been shown to be closely related to *P. chilensis* or *P. gilmartiniae.* In fact, they were both more closely related to *P. boliviensis* in both the nuclear and chloroplast phylogenies by Jabaily and Sytsma 2010. In the AFLP phylogeny by Jabaily and Sytsma (2013)

P. yakespala fell sister to the Yellow *Puya* group but *P. raimondii* was in the Northern Andean group. Our new finding suggests an interesting biogeographic history for *Puya*.

P. yakespala is endemic to a small, high-elevation region isolated from other *Puya,* on the Bolivian/Argentine border. It belongs to central Andean *Puya* but is a notably distinct entity due to both its isolation geographically and its relatively uncommon yellow flowers. Lack of blue/purple/black pigment in unusual in *Puya*. *Puya raimondii* has white flowers, lacking blue/purple/black pigment, and also lacking any visible yellow pigment (Hornung-Leoni et al., 2013). It is also geographically disjunct from the other three species, occurring in the northern Andes. *P. raimondii* is the largest *Puya* by far, and is the only fully-semelparous *Puya* known, found exclusively at extremely high elevations. But it does share sterile-tipped inflorescences with *P. chilensis* and *P. boliviensis.* Morphologically, *P. boliviensis* is very similar to *P. chilensis.* They share flower color and shape, glabrous leaves on the abaxial surface, and sterile-tipped inflorescences (Smith and Downs, 1974).

Schulte et al. (2010) found admixture in *P. gilmartiniae* samples that indicated that it was a hybrid between *P. chilensis* and *P. boliviensis.* In 1990, Varadarajan hypothesized the same thing, based on observations of its intermediate morphology. They proposed that *P. chilensis* and *P. boliviensis* lived in sympatry in the past, although *P. boliviensis* is now confined to the Atacama Desert and is the only *Puya* that lives there.

The PHYC locus places *P. chilensis* and *P. gilmartiniae* in Yellow *Puya*, sister to Core *Puya*, but the g3pdh locus embeds them in Core *Puya* still as close relatives, suggesting that the nucleus is heterogeneous, a mix of Chilean ancestry and central Andean ancestry. The ED clade is located near the base in all three phylogenies, grouping with central Andean taxa rather than northern Andean taxa. These results do not support step 1 of Jabaily and Sytsma's (2010) hypothesis. They show that *P. chilensis* and *P. gilmartiniae* are more closely related to Core *Puya*. They also support a distinct evolutionary history for *P. boliviensis.*

Two explanations for the close relatedness of taxa in the ED clade are relatively parsimonious. The first is a potential vicariance history for this clade; one in which evolution occurred because

an existing habitat range was severed by a new barrier, segregating portions of the lineage with no continued gene flow. In this scenario a yellow-flowered lineage was once wide-spread in South America and the only relicts remaining are the members of the ED clade. In the early Miocene a relatively homogenous woodland extended across southern South America and the species in it at the time had a broader climatic tolerance (Hinojosa & Villagrán, 2005), which supports the plausibility of a widespread Yellow *Puya* lineage. In this case, the ED clade could be an extreme example of *Puya* as an elevationally-mobile group that spread steadily from south to north but fluctuated freely in elevation. The branches in the ED clade are relatively long, so it is possible that with greater sampling, other members of this clade would be identified.

The other explanation involves recolonization of Chile by Yellow *Puya*. *Puya chilensis, P. gilmartiniae,* and *P. boliviensis* could be of a more derived origin, having evolved in the central Andes along with other extant central Andean taxa and later recolonized Chile during one or two dispersal events. It is likely that two recolonization events occurred because *P. boliviensis* and the ED clade have distinct lineages, and given the disjunct habitat range and morphology of *P. boliviensis* from *P. chilensis* and *P. gilmartiniae.*

Either explanation requires that changes be made to the working hypothesis. Figure 8 is a diagram of the updated hypothesis. Blue *Puya* is the ancestral *Puya* lineage. This is supported by biogeographic analysis by Jabaily and Sytsma (2013), which identified Chile as the center of origin for the genus *Puya*, and by the PHYC and g3pdh evidence that Blue *Puya* are the most basal taxa. In step 1, the ancestral *Puya* lineage splits into the Blue *Puya* ancestor and the Yellow *Puya* / Central Andean ancestor. Northern Andean *Puya* is assumed to be derived from central Andean lineages based on its phylogenetic position, the history of Andean uplift, and the biogeographic analysis by Jabaily and Sytsma (2013). Before step 2, evolution occurs to differentiate the Blue and Yellow *Puya* lineages. The chloroplast retains more similarity to the ancestral Blue *Puya* genome than the nucleus does. In step 2, Yellow *Puya* and central Andean lineages split. The blue chloroplast represents this ancestral chloroplast genome and the differently colored solid bars represent an ancestral nuclear genome of both Yellow *Puya* and central Andean taxa. G3pdh may be a locus that is conserved from before the split of Yellow *Puya*. Whether *P. chilensis, P. gilmartiniae*, and *P. boliviensis* are relicts or recolonizers, g3pdh

is like a synapomorphic (shared) character for Yellow *Puya* and central Andean taxa, and an autapomorphic (unique) character for the ED clade; while PHYC is like an autapomorphic character for Blue *Puya,* Yellow *Puya* and Core *Puya*, separately; and the concatenated cpDNA loci are autapomorphic for Chilean *Puya. Puya boliviensis* must have undergone chloroplast capture with a central Andean lineage in order to share nuclear ancestry with Yellow *Puya* and chloroplast ancestry with central Andean taxa (Fig. 8, step 4). The two arrows pointing down represent where recolonization of Chile may have occurred. Arrows pointing up represent different times when central Andean lineages may have been extirpated from Chile. Step 3 remains the same, with *P. alpestris* hypothesized to be a homoploid hybrid species.

Only the time-calibrated phylogeny shows structure in the ED clade. It shows *P. chilensis* as the most basal lineage in the ED clade, splitting off about 1.28 Ma (Fig. 4). Then *P. yakespala* split about 0.74 Ma, and *P. raimondii* and *P. gilmartiniae* split from each other about 0.32 Ma. These date estimates are later than the estimated origin of the MTC (3.2 - 5.3Ma), and later than the beginning of the most recent Andean uplift (3.6 Ma). They are, however, consistent with a period of major climatic fluctuation caused by glacial cycles which intensified during the Quaternary (starting about 1.8 Ma) (Arroyo, 1995). In addition, the topology of this clade is incongruent with that of the PHYC and chloroplast phylogenies by Jabaily and Sytsma (2010) which show that *P. chilensis* and *P. gilmartiniae* are more closely related to each other than to *P. yakespala* or *P. raimondii*.

It is important to note that our sampling included both northern and central Andean taxa and that northern Andean taxa occupied the most derived position, corroborating the finding by Jabaily and Sytsma (2013) that *Puya* colonized the northern Andes last.

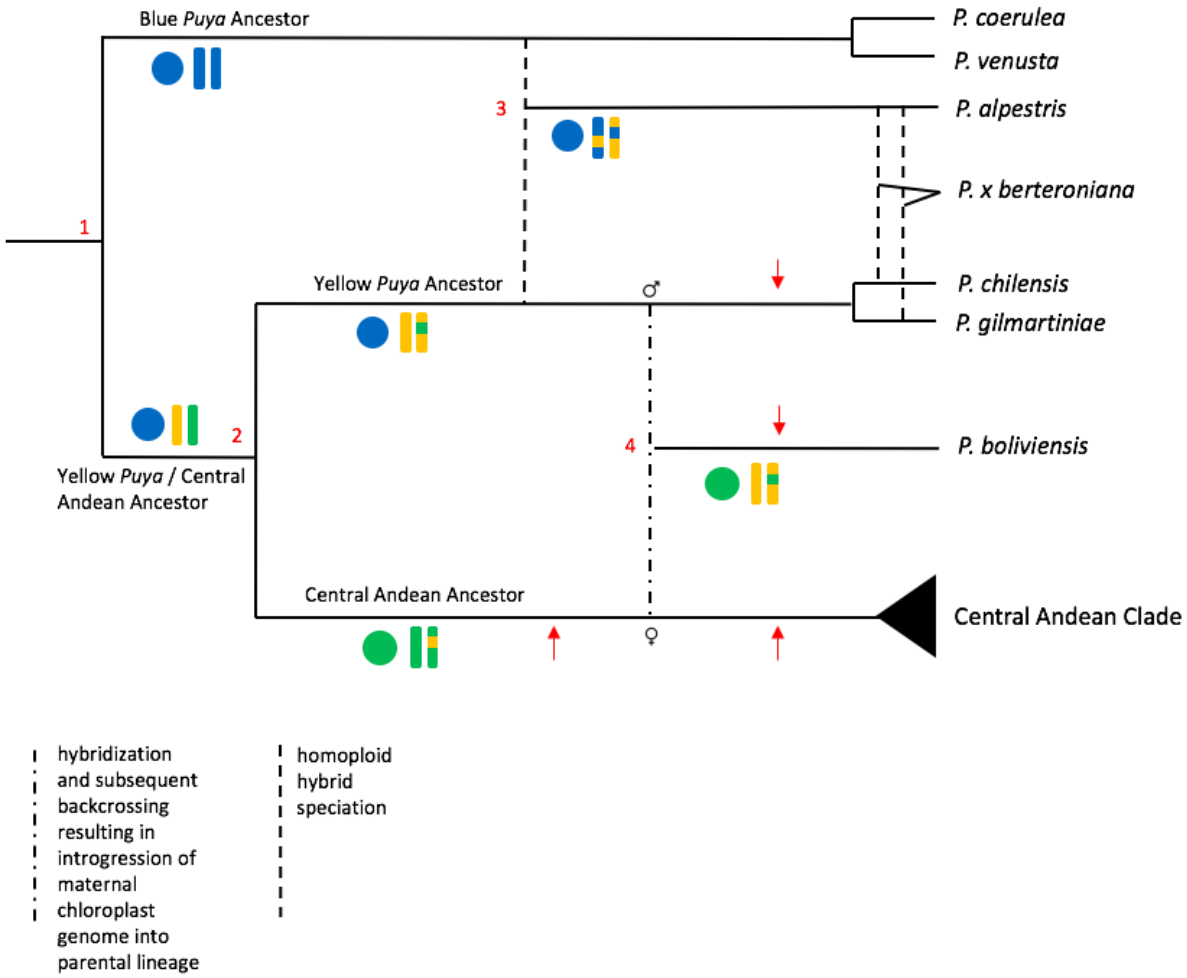

Figure 8. New hypothesis of molecular evolution in Chilean group. Adapted from Jabaily and Sytsma (2010) with additional hybridization inferred from Schulte et al., (2010) and Zizka et al., (2013), and finally modified with evidence from g3pdh phylogeny. Events of interest are numbered in red. Circles represent the chloroplast genome. Bars represent the nuclear genome. Down arrows represent proposed recolonization of Chile. Down arrows represent proposed extirpation from Chile.

Chloroplast Dated Phylogeny

As expected, this analysis followed the maximum likelihood analysis in Jabaily and Sytsma (2010) which showed Chilean *Puya* to be monophyletic except for *P. boliviensis.* And, as in the cpDNA phylogeny (Jabaily and Sytsma, 2010) Blue *Puya* and Yellow *Puya* do not form a clade together. Interestingly, these analyses show the closest relative of *P. berteroniana* to be *P. chilensis*, even though *P. berteroniana* is now not recognized as a distinct species. The g3pdh BEAST analysis showed *P. venusta* splitting from *Puya* first, at 10.04 Ma, and *P. alpestris* splitting next, at 8.05 Ma. However, in the chloroplast analysis these species are sister and only

split from each other 0.56 Ma. This is a major incongruence in the perceived amount of molecular different between the two loci and serves as strong support for the chloroplast capture hypothesis by Jabaily and Sytsma's (2010).

Nuclear and chloroplast incongruences

Historically, chloroplast loci have been used for molecular phylogenetics, but it is clear now that plastid data is not sufficient at the species level. Major clades (Bräuchler et al., 2010) and strongly-supported clades (Doyle & Gaut, 2000) can generally be resolved accurately using any locus. However, a chloroplast phylogeny only shows the maternal history because the chloroplast genome is inherited maternally and not bi-parentally (Nashima et al., 2015). Nuclear phylogenies show a biparental, and more biologically accurate, evolutionary history. As Folk et al. point out, most angiosperm molecular phylogenies in existence today should be retested because chloroplasts only tell half of the story (Folk et al., 2018). Another benefit of using nuclear loci is that different loci can be treated as independent datasets, as long as the loci do not belong to the same gene family. That is not true for chloroplast loci, which experience concerted evolution (Small et al., 2004), and are not independent.

Incongruences between nuclear phylogenies or between a chloroplast and a nuclear phylogeny hint that interesting evolutionary processes have occurred outside of the standard bifurcation of species. The use of nuclear loci comes with many difficulties, but if these can be sorted out we further our knowledge of how evolution works in the natural world. The first difficulty is that genes can occur in multiple copies. This introduces the issue of making sure orthologous, rather than paralogous regions are sequenced. There is still no straightforward or reliable process for determining orthology so there is always a risk that any phylogeny is not completely orthologous (Doyle and Gaut, 2000). The next issue is that of heterozygosity. Learning the biparental history of a group is important, but it comes with the challenge of inferring a phylogeny from sequences that may vary as a result of allelic diversity rather than just evolution. This is of greater concern at the population level, but it is still a factor at the species and generic levels (Small et al., 2004). Incomplete lineage sorting is a third issue. If a locus is polymorphic, the gene tree may not reflect the species tree accurately. Incomplete lineage sorting often occurs in conjunction with

rapid radiation events because those events are often caused by gene duplication (Yuan & Olmstead, 2008). *Puya* is known to have undergone such a rapid speciation event (Jabaily and Sytsma, 2013). Because there is no time-efficient way to know if a locus is polymorphic for a clade, it is possible that the use of any locus may result in issues with incomplete lineage sorting and that must be taken into account.

Yuan and Olmstead et al. (2008) show that the use of a single nuclear gene, no matter the length, can be misleading in the phylogenetic reconstruction it yields. Nuclear genes are subject to lineage sorting which may remain incomplete at the time of sampling, especially in rapidly evolving groups and when the focus is on low-level taxonomic relationships. Depending on the locus sampled, incomplete lineage sorting can result in a gene tree that is an inaccurate representation of the species tree. The additional data from g3pdh allows helps mitigate these issues by providing another source for comparison.

Conclusion

Novel findings

Phylogenetic reconstruction from the g3pdh locus yielded three novel findings. First, Yellow *Puya* was embedded within Core *Puya*, suggesting a closer history with those taxa than with Chilean *Puya*. Second, Blue *Puya* was found to be non-monophyletic. *Puya venusta* and *P. coerulea* are sister to Core *Puya*, and *P. alpestris* is equally related to *P. venusta, P. coerulea*, and to Core *Puya*. Even with low support, this remains a notable and interesting finding.

The third significant novel finding was the identification of a new clade comprising some Chilean taxa and some Core *Puya* taxa. In the Elevational Disjunct clade two yellow-flowered Chilean species are closely related to two central Andean species which were never before shown to be closely related to each other either. *P. raimondii* occurs exclusively at high elevations from Bolivia to central Peru, and *P. yakespala* occurs in a small range on the Bolivian/Argentine border, east of the Atacama Desert, and separated from Chile by a high part of the Andes that no *Puya* cross. So, I propose a new hypothesis about the origin of *P. chilensis*

and *P. gilmartiniae.* A lineage of Yellow *Puya* from Chile, and with a chloroplast genome similar to Blue *Puya,* was once widespread across southern and central South America. Then, due to some ecological and geological event, Yellow *Puya* died off in most parts and only the three Chilean yellow-flowered lineages we know of today persisted. Alternatively, after the die-off, some yellow lineages re-colonized Chile and then died off elsewhere. *P. raimondii* and *P. yakespala,* and potentially other, unsampled taxa as well, are the closest relatives of Chilean Yellow *Puya* because they all share a central Andean yellow-flowered ancestor that was once a widespread *Puya* lineage.

Corroborating findings

The position of *P. mima* is of note. It was at the base of Core *Puya* in all three phylogenies, and with high support. *P. mima* occupied the same position in the cpDNA phylogeny by Jabaily and Sytsma (2010) in a highly-supported polytomy at the backbone of Core *Puya.* In their PHYC phylogeny, *P. mima* was not sampled but *P. angusta* fell sister to Core *Puya* in its place, whereas it was embedded in Core *Puya* in the chloroplast phylogeny. Analyzing this interesting finding is outside the scope of this paper but should be followed up on in a subsequent publication.

Future research directions

This study raises many questions about the systematics and biogeographic history of *Puya*. Given the close connection that the ED clade suggests between central Andean *Puya* and Yellow *Puya,* greater sampling within central Andean Puya is needed to answer the following: Are there other central Andean yellow-flowered taxa that group with the ED clade? (It does not make sense that the closest relatives of *P. chilensis* are the taxa in the ED clade. Greater sampling might reveal that they are not, in fact, its closest relative.) Are there non-yellow-flowered central Andean taxa that group with the ED clade, or that do not group with the ED clade? How many taxa comprise the ED clade and where are they found? What geological events separated them and why did they persist when other yellow-flowered taxa did not? The finding that Blue *Puya* may be paraphyletic prompts future studies to look for a corroboration or to show that this may not be accurate. And it remains to be clarified what makes Chile a center of diversification.

The g3pdh topology and previous work in the genus also raise questions about hybridization. What are the pre- and post-zygotic barriers and how widespread are they in the genus? This question leads to questions about pollination. Are hummingbirds the pollinators of most species of *Puya*, as they are of some (Hornung-Leoni et al., 2013)? Are hummingbirds their only pollinators? Is there pollinator specificity in areas where *Puya* exists sympatrically?

Krapp et al. (2014) suggest an interesting biological source for incongruences seen in plastid versus PHYC phylogenies in their Bromeliad phylogeny focused on the genus *Dyckia*. They point out that chloroplasts are inherited only via the seed (maternally), while nuclear genes can be inherited via pollen (paternally). Pollen can travel much farther with animal pollinators (hummingbirds and insects) than the comparatively limited range of a wind-dispersed seed so the evolution of the two types of genetic information would occur at different paces. *Puya* would experience the same phenomenon because it is also hummingbird pollinated and wind-dispersed. This hypothesis about the biological connections of the incongruence studied here should be tested.

The use of g3pdh to build the Chilean *Puya* phylogeny yielded useful insight but there are limitations to phylogenetic analysis that uses one locus at a time, as discussed above. What is clearly needed is a molecular phylogeny that represents the species phylogeny. Hyb Seq will provide that phylogeny. R. Jabaily and J. Aguirre-Santoro are in the process of producing a Hyb Seq phylogeny of *Puya*. This next generation technique samples from 353 loci for each taxon and will provide a chloroplast topology, a nuclear topology, and a combined topology, all robustly supported with hundreds of loci. They have also sampled 110 taxa from across the genus including all the Chilean taxa discussed here, so many of the phylogenetic questions will be answered in the coming years. Hyb Seq will allow for direct comparison between a robust chloroplast phylogeny, and a comprehensive nuclear phylogeny that will take in to account many different regions of the genome and approximate the species phylogeny much more closely than anything previously achieved.

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Appendix

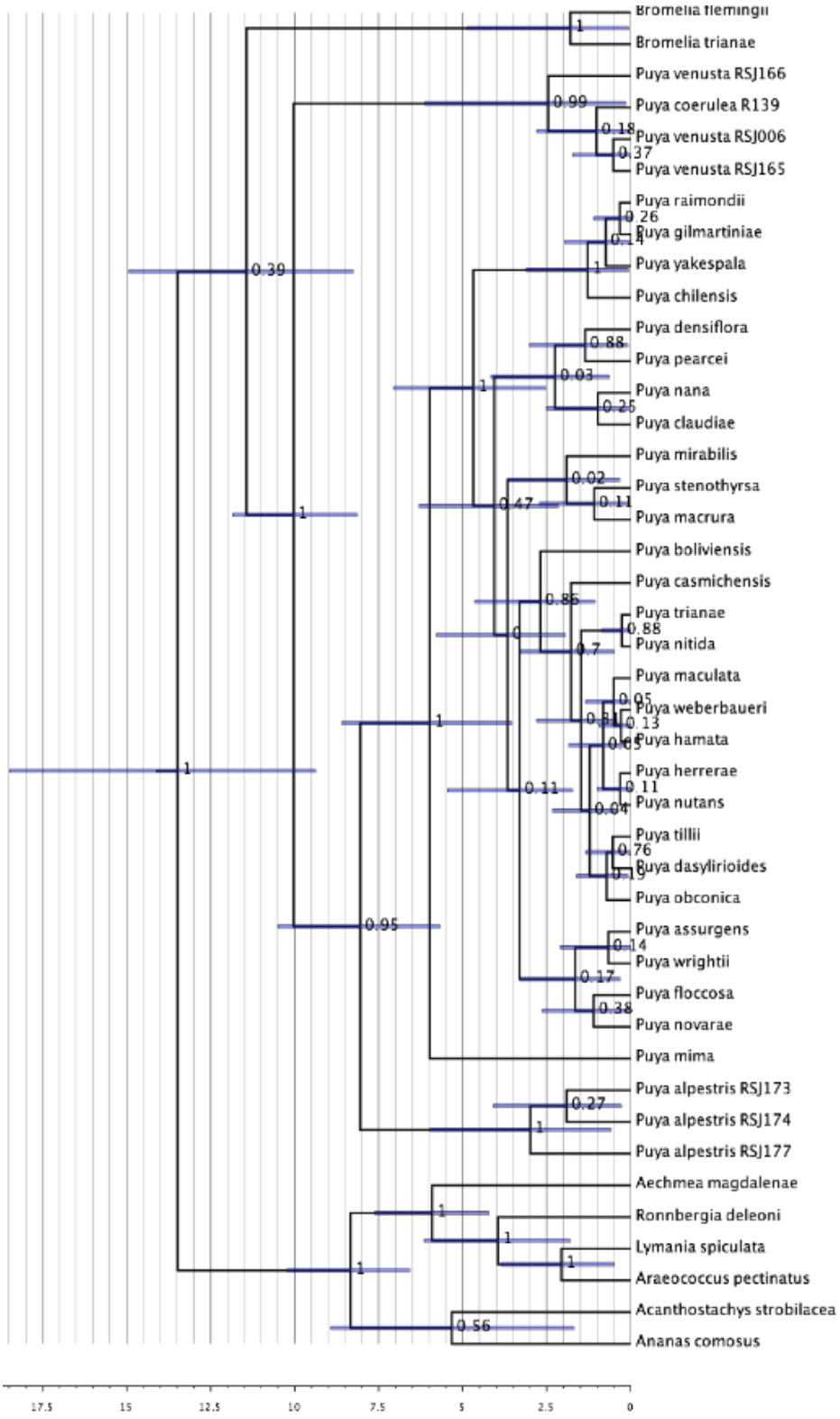
A. Table of Accessions

Collection Number	Subfamily	Taxon	Author	Collector	Elevation	Source
174	Puyoideae	*Puya alpestris*	(Poeppig) Gay	Rachel Schmidt Jabaily	350m	
177	Puyoideae	*Puya alpestris*	(Poeppig) Gay	Rachel Schmidt Jabaily	26m	
173	Puyoideae	*Puya alpestris*	(Poeppig) Gay	Rachel Schmidt Jabaily	41m	
26	Puyoideae	*Puya assurgens*	L.B. Smith	Rachel Schmidt Jabaily	-	Huntington Bot. Gar.
N/A	Puyoideae	*Puya boliviensis*	Baker	M. Rosas	-	M. Rosas 3351 (WIS)
223	Puyoideae	*Puya casmichensis*	L.B. Sm.	Rachel Schmidt Jabaily	2680m	
73	Puyoideae	*Puya stenothyrsa*	(Baker) Mez	Rachel Schmidt Jabaily	1850m	
172	Puyoideae	*Puya chilensis*	(Phil.) Baker	Rachel Schmidt Jabaily	296m	
65	Puyoideae	*Puya claudiae*	Ibisch	Rachel Schmidt Jabaily	1570m	
57	Puyoideae	*Puya coerulea*	Lindley	Rachel Schmidt Jabaily	-	Huntington Bot. Gar.
N/A	Puyoideae	*Puya dasylirioides*	Standl.	J. Grant	-	Field: 2141915
213	Puyoideae	*Puya densiflora*	Harms	Rachel Schmidt Jabaily	3000m	
N/A	Puyoideae	*Puya floccosa*	E. Morren	Rachel Schmidt Jabaily	-	Marie Selby Bot. Gar.
169 A	Puyoideae	*Puya gilmartiniae*	G.S.Varad. & Flores	Rachel Schmidt Jabaily	161m	
90	Puyoideae	*Puya hamata*	L.B.Sm.	Rachel Schmidt Jabaily	3760m	
212	Puyoideae	*Puya herrerae*	Harms	Rachel Schmidt Jabaily	3402m	
227	Puyoideae	*Puya macrura*	Mez	Rachel Schmidt Jabaily	2594m	
120	Puyoideae	*Puya maculata*	L.B.Sm.	Rachel Schmidt Jabaily	3642m	
228	Puyoideae	*Puya mima*	L.B.Smith & Read	Rachel Schmidt Jabaily	2594m	
153	Puyoideae	*Puya mirabilis*	(Mez) L.B. Sm.	Rachel Schmidt Jabaily	1463m	
62	Puyoideae	*Puya nana*	Wittm.	Rachel Schmidt Jabaily	1770 m	
179	Puyoideae	*Puya nitida*	Mez	Rachel Schmidt Jabaily	3496m	
156	Puyoideae	*Puya novarae*	Varadarajan ex Gomez Romero & A.Grau	Rachel Schmidt Jabaily	4012m	
117	Puyoideae	*Puya nutans*	L.B.Sm.	Rachel Schmidt Jabaily	3005m	

106	Puyoideae	*Puya obconica*	L.B.Sm.	Rachel Schmidt Jabaily	2451m	
38	Puyoideae	*Puya pearcei*	(Baker) Mez	Rachel Schmidt Jabaily	-	Huntington Bot. Gar.
229	Puyoideae	*Puya raimondii*	Harms	Rachel Schmidt Jabaily	4200m	
143	Puyoideae	*Puya tillii*	Manzan.	Rachel Schmidt Jabaily	2937m	
183	Puyoideae	*Puya trianae*	Baker	Rachel Schmidt Jabaily	3669m	
6	Puyoideae	*Puya venusta*	(Baker) Phil.	Rachel Schmidt Jabaily	-	Huntington Bot. Gar.
166	Puyoideae	*Puya venusta*	(Baker) Phil.	Rachel Schmidt Jabaily	60m	
165	Puyoideae	*Puya venusta*	(Baker) Phil.	Rachel Schmidt Jabaily	3m	
217	Puyoideae	*Puya weberbaueri*	Mez	Rachel Schmidt Jabaily	-	Huntington Bot. Gar.
39	Puyoideae	*Puya wrightii*	L.B. Sm.	Rachel Schmidt Jabaily	-	Huntington Bot. Gar.
157	Puyoideae	*Puya yakespala*	A.Cast.	Rachel Schmidt Jabaily	3628m	

Outgroups:				
Acanthostachys strobilacea				1. Sass, C. and Specht, C. D. (2010) Phylogenetic estimation of the core Bromelioids with an emphasis on the genus Aechmea (Bromeliaceae). Mol. Phylogenet. Evol. 55 (2), 559-571
Aechmea magdalenae				2. Aguirre-Santoro,J., Michelangeli,F.A. and Stevenson,D.W. (2016) Molecular phylogenetics of the Ronnbergia Alliance (Bromeliaceae, Bromelioideae) and insights into their morphological evolution. Mol. Phylogenet. Evol. 100, 1-20.
Ananas comosus				2.
Araeococcus pectinatus				1.
Bromelia flemingii				2.
Bromelia trianae				2.
Lymania spiculata				1.
Ronnbergia deleonii				2.

B. Molecular dated phylogeny of G3pdh with Bayesian posterior probabilities. Scale axis shows age.



C. Molecular dated phylogeny of *matK, trnS-trnG,* and *rps16* chloroplast regions from Jabaily & Sytsma, 2010 with Baysian posterior probabilities. Scale axis shows age.