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Phylogeny, Phenology, and Foraging Breadth of *Ashmeadiella* (Hymenoptera: Megachilidae)

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Abstract

Ashmeadiella Cockerell (Megachilidae: Osmiini) is a bee genus endemic to North America, with greatest richness in arid and Mediterranean regions of the southwestern United States. Species relationships of *Ashmeadiella* were last analyzed in the 1950s, when Robert Sokal and Charles Michener developed a novel statistical clustering method for classification called numerical taxonomy. To revisit the taxonomic groups they established, we built a molecular phylogeny including all five subgenera. Furthermore, we assembled life history data to lay the foundation for future conservation programs for these bees. We chose three aspects of bee biology that can inform conservation strategies: documenting periods of the year adult bees are flying, assembling data for the flowers each species visits, and compiling the localities and ecoregions where each species is reported. Our results suggest that some *Ashmeadiella* species may need to be synonymized and that the subgenera should be revised due to non-monophyly. We therefore propose synonymizing the subgenera *Cubitognatha* and *Chilosima* with *Arogochila*. Biological data from published collection records reveal that adult flight periods range from a few months to 11 mo; most species utilize floral resources from multiple plant families; and, over half of the species have ranges extending into the Mojave Desert.

Key words: bee, diversity, ecology, phylogenetics, pollinator

Bees (Hymenoptera: Anthophila) are considered crucial to ecosystem function for their pollination services in both natural and human-modified settings including agricultural production (Klein et al. 2007, Hoehn et al. 2008, Kleijn et al. 2009, Winfree et al. 2018). Still, as with most invertebrates, many bee species are poorly documented (Minckley and Ascher 2013, Goulson 2019) or undescribed (Packer and Taylor 1997, Buchmann and Ascher 2005, Batley and Hogendoorn 2009); even more lack molecular data to aid in determining species relatedness (Packer and Taylor 1997). A lack of data on native bees makes it difficult to monitor species decline or distributional trends (Buchmann and Ascher 2005, Goulson 2019).

It is critical to assemble data on the life history, ranges, and evolutionary relationships of native bees for both scientific inquiry and legal protections; although no specific data are required in deciding which species could be protected by the Endangered Species Act (Doremus 1997, Fallon 2007), as there are no set criteria for listing (Easter-Pilcher 1996). Species in the United States that have more data—including historical ranges, phenology, genetic distinctiveness, and classifications—can be more easily evaluated for potential legal protections. For example, in another charismatic insect pollinator group, the butterflies, every known species in the United States and Canada has a partial or complete genome sequence (845 species; Zhang et al. 2019), and most butterflies in Canada and the United States have at least minimal biological information of suitable host plants and flight periods. Furthermore, the United States currently lists 34 butterfly species as threatened or endangered (4% of the total U.S. species) under the Endangered Species Act. In contrast, a mere eight bees of the roughly 4,000 species occurring in the United States are listed at this time in 2020 (~0.2%). One explanation for this discrepancy is that butterflies truly are in greater danger of

extinction, but it is quite likely that bees, though also a charismatic group, lack much of the data that butterfly species have in respect to conservation. Data on bee species relationships, distributions, and biologies will help to identify potentially threatened species.

Ashmeadiella Cockerell, a genus in the family Megachilidae, is one of the many poorly studied groups of bees. It is a diverse genus endemic to North America, with 61 described species (Table 1) as well as a few known undescribed species (Carril et al. 2018). We consulted published dictionaries and native speakers of Hopi (Glosbe 2020), Jemez-Pueblo (Clarence Toya, personal communication, 2020), Nahuatl (Eduardo de la Cruz, personal communication, 2020; Sullivan et al. 2020), Navajo/ Diné (Sue Whitey, personal communication, 2020; Yazzie et al. 2007), Shoshone (Russel Jones, personal communication, 2020), and native Spanish speakers from Chihuahua, Mexico and Mexico City (Armida Valezuela, personal communication 2020), and found no specific names for *Ashmeadiella* or the subfamily Megachilinae, which indicates that in 1897, Cockerell was likely one of the first people to identify this group of bees.

To date, classification of *Ashmeadiella* species and subgeneric groups is based on morphological similarity with little to no genetic data to corroborate hypotheses of species relationships. Species within the genus are medium to small, robust bees that are slenderer than many other Megachilidae (Michener et al. 1994, Michener 2007). Species exhibit both polylectic and oligolectic foraging behaviors (Michener 1939, Hurd and Michener 1955, Yanega 1994, Wilson and Carril 2015). Nesting sites include tunnels in wood and hollowed stems, in areas under rocks, in snail shells, and excavated terrestrial tunnels (Michener 1939, Yanega 1994, Wilson and Carril 2015). As with many bee groups, there has been flux in the circumscription and membership of the genus. For example, the subgenus *Isosmia* Michener and Sokal was previously included in *Anthocopa* Lepeletier and Serville (Hurd and Michener 1955) but was transferred to *Ashmeadiella* while the rest of the '*Anthocopa*' from the Americas were included in a newly recognized genus: *Atoposmia* Cockerell (Griswold and Michener 1997).

Most *Ashmeadiella* are reported from xeric and Mediterranean environments in the Southwest, with a few species occurring in the central and eastern United States, as well as western Canada and southern Mexico (Michener 1939). The arid ecoregions where *Ashmeadiella* are most diverse are currently under high rates of land conversion for green energy infrastructure and urban sprawl (Brooks et al. 2002, Lovich and Ennen 2011, McCoshum and Geber 2020). With the current lack of ecological data for each species, it is difficult to predict or study how bees are responding to these transitions; however, habitat loss for ten species of *Ashmeadiella* has been modeled for parts of the Mojave and Sonoran deserts (McCoshum and Geber 2020). Part of determining the ecology and conservation needs of *Ashmeadiella* requires understanding phylogenetic relationships and ensuring there are data to illustrate that described species are distinct, so that distributions, phenology, and other data can be used in evaluating population health. By adding molecular data to our understanding of each species, we can encourage approaches to conserve phylogenetic diversity. A phylogenetic framework can also be used to hypothesize rare species' life histories and responses to environmental changes, as evidence suggests that phylogenetic groups have similar functional traits like nesting and flower preference, as well as responses to land-use change (Almeida 2008, Williams et al. 2010, Rader et al. 2014).

The phylogeny of *Ashmeadiella* is also interesting from a historical perspective, due to its groundbreaking role in the taxonomic realm. Robert Sokal and Charles Michener published some of the first papers detailing a statistics-based technique that later became known as numerical taxonomy (Michener and Sokal 1957, Sokal and Michener 1958). Numerical taxonomy required large numbers of unbiased characters to algorithmically cluster organisms together based on similarity; it was meant to be a means of classification, but not of establishing phylogenetic relationships (Vernon 1988). A subset of megachilid bees, including several *Ashmeadiella* species, were used to illustrate their system of numerical taxonomy, but their taxonomic results have not been explored since. Our paper reexamines *Ashmeadiella* using a molecular phylogeny to evaluate morphologically defined subgeneric groups and species relationships. Additionally, to help inform future conservation strategies, we analyzed the phenology of each species to elucidate active flight periods to investigate whether any species are temporally isolated throughout the year. Further, we investigated each species' known foraging breadth based on published records to elucidate floral associations and identify species which have little floral data.

Methods

Phylogeny

Ashmeadiella specimens sequenced include 33 described species and three undescribed species, representing all five subgenera. Specimen vouchers for this study are deposited in the U.S. National Pollinating Insects Collection (Logan, Utah) and in the collection of Robert L. Minckley at the University of Rochester (Supp Table 1 [online only]). Species were determined or confirmed by Kim Huntzinger, T. Griswold, and S. Bossert. We sequenced 79 *Ashmeadiella* specimens for this study. To increase taxon sampling, additional sequences were downloaded from GenBank (Supp Table 1 [online only]). GenBank sequences included five *Ashmeadiella* individuals and 29 other megachiline taxa sampled as outgroups.

DNA Extraction, Amplification, and Alignment

DNA was extracted from one to three legs of pinned specimens using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). Three gene regions were chosen for use in phylogenetic reconstruction, based on their historical success in bee phylogenies (Danforth 1999, Mardulyn and Cameron 1999, Danforth et al. 2006): an 825 bp region of the mitochondrial gene cytochrome oxidase subunit 1 (COI), an 800 bp region of the nuclear gene conserved ATPase domain (CAD), and a 645 bp region of the nuclear gene long-wavelength rhodopsin (OPS). In addition to commonly used primers, we developed a new internal primer for CAD (Supp Table 2 [online only]). The CAD region required two PCR reactions, as it is a composite of two overlapping subregions which were amplified and sequenced separately.

After amplification, PCR products were purified using a sequence of protocols involving Exonuclease I (New England Biolabs, Ipswich, MA), Shrimp Alkaline Phosphatase (GE Healthcare Biosciences Corp., Piscataway, NJ), and the Agencourt CleanSEQ kit (Beckman Coulter Genomics, Morrisville, NC). Sequencing was done on an Applied Biosystems Automated 3730 DNA Analyzer using Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase (Cornell, Ithaca, NY). Sequences are available in GenBank under the following accession numbers: COI: MW726116 - MW726192, CAD: MW760001 - MW760079, and OPS: MW760080 - MW760151.

Sequence Alignment and Metrics

Sequencher v5.4.6 (Gene Codes Corp, Ann Arbor, MI) was used to clean and verify sequences. All genes were aligned in the online

Table 1. List of described species and museum species identifications for specimens with: determination authority, Integrative Taxonomy Information System numbers, inclusion in the phylogeny (see SuppTable 1 [online only] for more information and GenBank accession numbers), total number of digitized records with GPS data, collection date data, number of unique collection dates, and number of specimens with floral records. Superscripted names include the following synonymized taxa. 1. *Ashmeadiella astragali*, 2. *A. basalis* & *A. echinocerei*, 3. *A. coquilletti* & *A. sierraensis*, 4. *A. washingtonensis*, 5. *A. cismontanica*, *A. rubra*, & *A. rufiventris*, 6. *A. arizonensis*, and 7. *A. howardii*

Species name	Authority	ITIS number	Species included in our tree	With Geo data	With collection date data	Number of unique date data	With floral record
no species ID	Cockerell 1897	634023	-	1,903	-	-	146
A. altadenae	Michener, 1936	715333	N	14	27	7	1
A. aridula [1]	Cockerell 1902	715334	Y	1,766	1,475	456	36
A. australis	Cockerell 1902	715335	Y	407	342	63	5
A. barberi	Michener, 1939	715336	Y	4	5	5	0
A. bequaerti	Cockerell 1931	715337	N	54	176	40	0
A. bigeloviae	Cockerell, 1897	715338	Y	1,857	2,996	340	78
A. biscopula	Michener, 1939	715339	N	30	93	28	2
A. breviceps	Michener, 1939	715340	Y	333	575	339	16
A. bucconis	Say, 1837	715341	Y	1,718	1,360	374	79
A. cactorum [2]	Cockerell, 1897	715342	Y	2,786	1,738	325	65
A. californica [3]	Ashmead, 1897	715343	Y	1,088	1,358	686	61
A. cazieri	Michener, 1939	715344	Y	140	271	49	5
A. chumashae	Griswold, 1985	715345	N	0	5	5	0
A. clypeodentata	Michener, 1936	715346	Y	211	500	71	11
A. cockerelli	Michener, 1936	715347	N	4	13	5	0
A. crassa	Cockerell, 1924	715348	N	0	0	0	0
A. cubiceps	Cresson, 1879	715349	Y	174	251	57	7
A. danuncia	Ayala et al. 2015	none	N	0	0	0	0
A. difugita	Michener, 1939	715350	N	165	113	33	2
A. digiticauda	Cockerell, 1924	715351	N	1	6	2	0
A. dimalla	Michener, 1939	715352	N	0	3	1	0
A. erema	Michener, 1939	715353	Y	99	163	28	4
A. eurynorhyncha	Michener, 1939	715354	Y	92	74	14	1
A. femorata	Michener, 1936	715355	Y	341	421	79	7
A. floridana	Robertson, 1897	715356	N	21	47	12	0
A. foveata	Michener, 1939	715357	Y	213	186	58	12
A. foxiella [4]	Michener, 1939	715358	N	12	39	9	2
A. gillettei [5]	Titus, 1904	715359	Y	1,264	1,515	222	22
A. holtii	Cockerell, 1898	715360	Y	219	319	26	0
A. hurdiana	Michener, 1954	715361	Y	35	49	10	0
A. inyoensis	Michener, 1939	715362	N	51	87	23	10
A. lateralis	Michener, 1936	715363	N	1	3	1	1
A. leachi	Michener, 1949	715364	N	70	104	12	0
A. leucozona	Cockerell, 1924	715365	N	407	1,127	77	8
A. lutzi	Cockerell, 1930	715366	Y	48	41	7	2
A. mandibularis	Ayala et al. 2015	none	N	0	0	0	0
A. maxima	Michener, 1936	715367	N	41	107	23	3
A. meliloti	Cockerell, 1897	715368	Y	3,135	1,920	305	68
A. micheneri	Snelling, 1962	715369	N	28	62	4	2
A. microsoma	Cockerell, 1924	715370	N	0	0	0	0
A. neomexicana	Cockerell, 1904	715371	N	80	88	4	0
A. occipitalis	Michener, 1939	715372	Y	245	588	108	16
A. opuntiae [6]	Cockerell, 1897	715373	Y	1,356	1,482	163	20
A. parkinsoniae	Parker, 1977	715374	N	13	30	3	3
A. pronitens	Cockerell, 1906	715375	N	41	99	32	2
A. prosopidis	Cockerell, 1897	715376	Y	141	470	53	16
A. rhodognatha	Cockerell, 1924	715377	Y	520	852	116	12
A. rubrella	Michener, 1949	715378	Y	539	744	64	4
A. rufipes	Titus, 1904	715379	Y	323	540	70	11
A. rufitarsis	Michener, 1939	715380	Y	161	267	19	4
A. salviae [7]	Michener, 1939	715381	Y	222	167	46	7
A. sangrita	Peters, 1972	715382	N	8	27	7	0
A. sculleni	Michener, 1939	715383	N	5	12	4	0
A. sonora	Michener, 1939	715384	Y	636	537	87	12
A. stenognatha	Michener, 1939	715385	N	27	8	2	0
A. stevensi	Michener, 1937	715386	N	2	9	3	1
A. timberlakei	Michener, 1936	715387	Y	715	1,032	207	35
A. titusi	Michener, 1939	715388	N	36	29	12	3
A. truncativentris	Michener, 1951	715389	N	19	15	3	1
A. vandykiella	Michener, 1949	715390	Y	89	47	11	0
A. xenomastax	Michener, 1939	715391	Y	395	390	72	11

portal of MAFFT v7 (Katoh and Standley 2013) and iteratively checked against chromatograms. Aligned genes were then verified by eye in Mesquite (Maddison and Maddison 2018), resulting in a few manual alignment modifications and edits to incorrect base calls. Ends were trimmed, and then intron and exon boundaries and codon reading frames were annotated. COI had 323 parsimony-informative sites (39.2% of the total 825 sites); CAD had 242 (23.7% of 1023 sites); and OPS had 249 (29.2% of 853). For COI, species distances were calculated using 'ape' v5.4.1 (Paradis and Schliep 2019) in R v3.6.1 (R Core Team 2020, RStudio 2020). Though our COI region is not the barcode region, we followed previous research parameters (Hebert et al. 2003) and corrected distances with the Kimura-two-parameter nucleotide substitution model and used a 3% threshold for circumscribing species boundaries.

Phylogenetic Analysis

Concatenated and individual gene trees were reconstructed using IQ-TREE v1.6.12 (Nguyen et al. 2015) using their web server (Trifinopoulos et al. 2016). The mitochondrial gene COI (92 individuals) was comprised of two subsets: positions 1 + 2 and position 3. Each nuclear gene was partitioned by exon and intron, and exons were partitioned into codon positions 1 + 2 as a separate subset from position 3. CAD (110 individuals), with one intron, was divided into five subsets, and OPS (103 individuals), with two introns, was also partitioned into five, with the two introns in one subset, and exon three combined with exon two, because it was only 21 nucleotides long. The concatenated dataset was comprised of 108 taxa (84 being *Ashmeadiella*) and 2701 sites. All gene subsets were retained, resulting in 12 partitions. For individual genes and the concatenated analysis in IQ-TREE, substitution models were automatically chosen, and free rate heterogeneity was used. IQ-TREE calculated edge-linked branch lengths (Chernomor et al. 2016) and gave branch support from ultrafast bootstrapping (Hoang et al. 2018).

Ashmeadiella Ecology

Digitized specimen label data were downloaded from the Global Biodiversity Index Facility (GBIF 2019) and Symbiota Collections of Arthropods Network (SCAN) (September 2019), and obtained directly from the American Museum of Natural History, Santa Barbara Museum of Natural History, UC Davis Bohart Museum of Entomology, and UC Riverside Entomology Research Museum in September 2019. Data for *A. barberi* Michener is from the USDA Bee Biology and Systematics Lab. As with most digital data entry, we could not verify species IDs or accuracy of data entered, so some species may have incorrect data incorporated in our analyses. We removed records with invalid names unless we could determine their correct species association (nine synonymized, two invalid: Table 1). The combined records are referred to as 'collections data' below.

Phenology and Forage

Collections data were used to isolate specimens with collection dates, which were then sorted by month, day, and year. To avoid yearly sampling bias where 1) many specimens were collected on a single day possibly by multiple collectors, and 2) repetitive collection reports were logged in multiple organizations or data platforms, we only counted one observation per day, per year for each species. These data were then used to estimate when the adults of each species are flying throughout the course of a year. We further investigated patterns between clades by comparing flight season length between clades using an ANOVA, and emergence dates using Welch's *t*-test. We filtered data which also had GPS coordinates and tested if there was latitudinal correlation with adult activity using Pearson Correlation Coefficient.

Using the collections data and reports from publications (Robertson 1928, Grant and Grant 1979, Grant and Hurd 1979, Yanega 1994, McIntosh 2005, Blair and Williamson 2008, Carril et al. 2018), we isolated specimens that had floral association data, to provide information on potential forage plants providing pollen or nectar. In some cases, there are taxonomic challenges in determining which plant species the bees were collected on, as plant names have been synonymized, or authors used incorrect classifications. For example, *A. meliloti* and *A. opuntiae* were recorded with the note '*Opuntia megacarpa*: southern California form of *O. discata*' (Grant and Grant 1979). In this case, *O. megacarpa* Griffiths (Cactaceae: Caryophyllales) has been synonymized with *O. engelmannii* Salm-Dyck, and *O. discata* Griffiths is a distinct species, so it is unclear which species these bees were collected from. In cases like these we only retained the plant genus data.

Many plants listed on collection records were not identified to species, so we counted undetermined plant species records only once per genus (Supp Table 3 [online only]). The majority of the data also lack bee sex determinations and whether the bees were observed collecting pollen or nectaring. For some plant species such as *Asclepias* (milkweed), it is clear bees were only nectaring and not collecting pollen, as pollen from these plants are packaged into pollinia (Theiss et al. 2007) that are not used by bees. However, for most reports no assumptions can be made. Males are known to be less selective when nectaring compared to females (Ne'eman et al. 2006, Smith et al. 2019), which makes determining forage breadth difficult, so we are using plant records only as a record of floral associations and not as a measure of host specificity or female nest provisioning.

The host plant records assembled for *Ashmeadiella* were used to visualize the bee-flower associations using a bipartite network. Bipartite network graphs show two sets of classes, where every member of one set can be connected to any member of the other set. They are commonly used for pollination networks, but we have not encountered them as a means to display the known flower breadth of a taxon of bees. These records should not be interpreted as bee diet data, but as floral associations. Due to the large number of plant species used by *Ashmeadiella* (>400, Supp Table 3 [online only]), we illustrate plant associations at the family level via a bipartite network analysis using the R package bipartite (Dormann et al. 2008).

Diversity Assessment

We used the collections data to map specimens having spatial coordinates in ArcMap10.3. Due to many species having fewer than 20 collection locations, species distribution models were not suitable. To estimate which ecoregions potentially have the greatest species richness of *Ashmeadiella*, we used the United States EPA Ecoregion Level III shapefile (EPA 2020). We chose the Ecoregion Level III because it is used as a guideline for government sponsored programs like Seeds for Success (Haidet and Olwell 2015), is based on environmental parameters that are likely to affect *Ashmeadiella* habitat suitability, and is available free of charge for use by researchers and the public. We added a presence column to the ecoregions shape file and created point shapefiles using GPS data for each species. Using 'Spatial Join' for each species layer, we were able to isolate ecoregions where *Ashmeadiella* species have been collected, then put a '1' in the presence column for occupied ecoregions, and '0' in un-occupied ecoregions. Spatial Join products were converted to rasters, then added together using 'Raster Calc' to create the final richness count.

To visually interpret the patterns of *Ashmeadiella* collecting and reported identifications over time, we plotted collection records of each species (filtered to one record per species per day, as above).

Results

Phylogeny

In the concatenated analysis, *Ashmeadiella* is strongly supported as monophyletic (Fig. 1). Nonetheless the monophyly of *Ashmeadiella* cannot be confidently confirmed based on individual gene trees due to inconsistencies in the relationship of the subgenus *Isosmia* Michener and Sokal and some outgroup genera (Supp Figs. 1–3 [online only]). Based on the CAD results, part of *Atoposmia*, *Osmia*, and *Hoplosmia* are grouped with *Isosmia*. The sister genus of *Ashmeadiella*, *Atoposmia*, may render *Ashmeadiella* paraphyletic, indicated by the differing topological patterns in each of the genes. The gene tree of COI provides no information because COI sequence data for *Atoposmia* is not available (Supp Fig. 1 [online only]). However, CAD results show *Ashmeadiella* as nonmonophyletic (Supp Fig. 2 [online only]). The six *Atoposmia* species are polyphyletic, though all nodes are weakly supported (bootstrap support [BS] = 30–56). One species (*At.* aff *daleae*) is within *Ashmeadiella*, branching after *Isosmia*, and sister to the rest of the genus. Lastly, OPS results show *Ashmeadiella* as monophyletic (Supp Fig. 3 [online only]) though the six *Atoposmia* are again not monophyletic, with five of the species forming a clade as the sister group to *Ashmeadiella*. The close relationship of *Atoposmia* and *Ashmeadiella* was previously found in a broad analysis having denser sampling of Osmiini, though including no representatives of the subgenus *Isosmia* (Praz et al. 2008).

The *Ashmeadiella* included in our phylogeny can be divided into three major clades (Figs. 1 and 2). Clade I is the sister clade to the rest of the genus (BS = 100) and consists of one subgenus, *Isosmia*. For *Isosmia*, we sampled two of the three described species and one undescribed species that has morphological characteristics diagnostic of the subgenus. Our results provide evidence for monophyly of the subgenus.

Clade II (BS = 100) consists of three subgenera: *Arogochila* Michener, *Chilosima* Michener and *Cubitognatha* Michener. The species relationships of *Arogochila* differ across the three gene trees, and there is low support for many interspecific branches. *Arogochila* is not a monophyletic subgenus in any of our analyses, due to the inclusion of two small subgenera, *Chilosima* and *Cubitognatha*. In the concatenated and COI phylogenies, *Chilosima* + *Cubitognatha* form a monophyletic group (Fig. 1, BS = 95; Supp Fig. 1 [online only], BS = 90). In the phylogenies built from CAD (Supp Fig. 2 [online only]) and OPS (Supp Fig. 3 [online only]), *Chilosima* and *Cubitognatha* are nested within *Arogochila*, though the two are not closest relatives of each other. The subgenus *Chilosima*, comprised of two species, is monophyletic in our analyses. The monotypic subgenus *Cubitognatha* is monophyletic in most analyses. We included three specimens of *A.* (*Cub.*) *xenomastax* Michener, which clustered together in all phylogenies except the CAD gene tree (Supp Fig. 2 [online only]).

Clade III (BS = 100) is composed solely of subgenus *Ashmeadiella* Cockerell. However, not all species of that subgenus clustered in this clade in our concatenated analysis. There are two species, *A.* (*Ash.*) *femorata* Michener and *A.* (*Ash.*) *rufitarsis* Michener, which have differing placements in individual gene tree analyses. In the concatenated phylogeny, *A.* (*Ash.*) *femorata* is placed as the sister to Clades II + III, and *A.* (*Ash.*) *rufitarsis* is placed as sister to Clade II. Otherwise, all subgenus *Ashmeadiella* species sampled form a monophyletic group in Clade III.

We examined species boundaries within the genus using information from COI distances. For COI, there are 27 species with two or more representatives, and all species fell below a 3% cutoff value for species limits. However, we have not sampled the full extent of the species' geographic distributions, which would help clarify species boundaries. The highest maximum intraspecific COI divergence for a monophyletic species in our tree was 2.65%, in *A.* (*Chi.*) *rhodognatha* Cockerell. However, genetic distances do not reveal evolutionary relationships—the concatenated phylogeny shows several instances where described species are not monophyletic, based on our data. There are three species pairs that formed mixed clades: *A.* (*Aro.*) *lutzi* Cockerell + *A.* (*Aro.*) *timberlakei* Michener (BS = 100), which together have a COI distance of 1.0%; *A. foveata* Michener + *A. vandykiella* Michener (BS = 100) with a combined distance of 1.35%; and *A.* (*Chi.*) *rhodognatha* + *A.* (*Chi.*) *holtii* Cockerell (BS = 100), which have a combined distance of 3.68%.

Phenology and Forage

Ashmeadiella species take flight throughout the year, with museum data showing most species flying in April, May, and June (Fig. 3, Supp Figs. 4 and 5 [online only]). Our analyses exploring latitudinal correlations show many species are collected earlier in southern latitudes, but some species, including *A. bequaerti* Cockerell, show no significant correlation with latitude and flight times (Supp Fig. 5 [online only]). Five species (all but one in Clade III (subgenus *Ashmeadiella*)) were collected multiple times in January: *A. bequaerti* Cockerell, *A. californica* Ashmead, *A. maxima* Michener, *A. neomexicana* Cockerell (subgenus *Arogochila*), and *A. sonora* Michener (Fig. 3, Supp Figs. 4 and 5 [online only]). Four of these species have GPS data which ranged in latitude from 19.7 to 37.5 (WGS 1984) or collections in January (Supp Fig. 5 [online only]).

Flight season length tends to be longer in Clade III than Clade II ($F = 14.7$, $P < 0.0005$) with an average of 82 d (Supp Figs. 4 and 5), but no significant differences were found between earliest emergence ($P > 0.1$). The species with the longest active flight periods spanning more than ten full months were *A. aridula* Cockerell, *A. bequaerti*, *A. bigeloviae* Cockerell, *A. californica*, *A. cazieri* Michener, *A. maxima*, *A. meliloti* Cockerell, *A. neomexicana*, and *A. sonora*. Clade II (*Arogochila*) has the three species which appear to have short flight seasons that are less than 2 mo: *A. foxiella* Michener and *A. lutzi* (Fig. 3, Supp Fig. 4 [online only]). There were not enough data to determine the flight periods for *A. crassa* Cockerell, *A. danuncia* Ayala, Griswold and Vergara, *A. dimalla* Michener, *A. lateralis* Michener, *A. mandibularis* Ayala, Griswold and Vergara, *A. micheneri* Snelling, *A. microsoma* Cockerell, *A. parkinsoniae* Parker, *A. sculleni* Michener or *A. truncativentris* Michener. The latest-emerging species is *A. sangrita* Peters, which emerges in August and flies as late as December (Supp Figs. 4 and 5 [online only]).

According to the available digital data, *Ashmeadiella* have been collected from plants of 44 families, 182 genera, and more than 400 species (Fig. 3, Supp Fig. 4 [online only], Supp Table 3 [online only]). The three plant genera with the highest number of associates were *Phacelia* Jussieu (Boraginales: Boraginaceae) with 18 bee species, and *Cryptantha* Lehm. ex G. Don (Boraginaceae) and *Melilotus* Millspaugh (Fabales: Fabaceae) each with 16 bee species. The plant families with the highest number of *Ashmeadiella* species are Fabaceae with 36 species of *Ashmeadiella*, Boraginaceae with 28, and Asteraceae (Asterales) with 26 (Fig. 3, Supp Tables 3 and 4 [online only]). The *Ashmeadiella* species with the highest

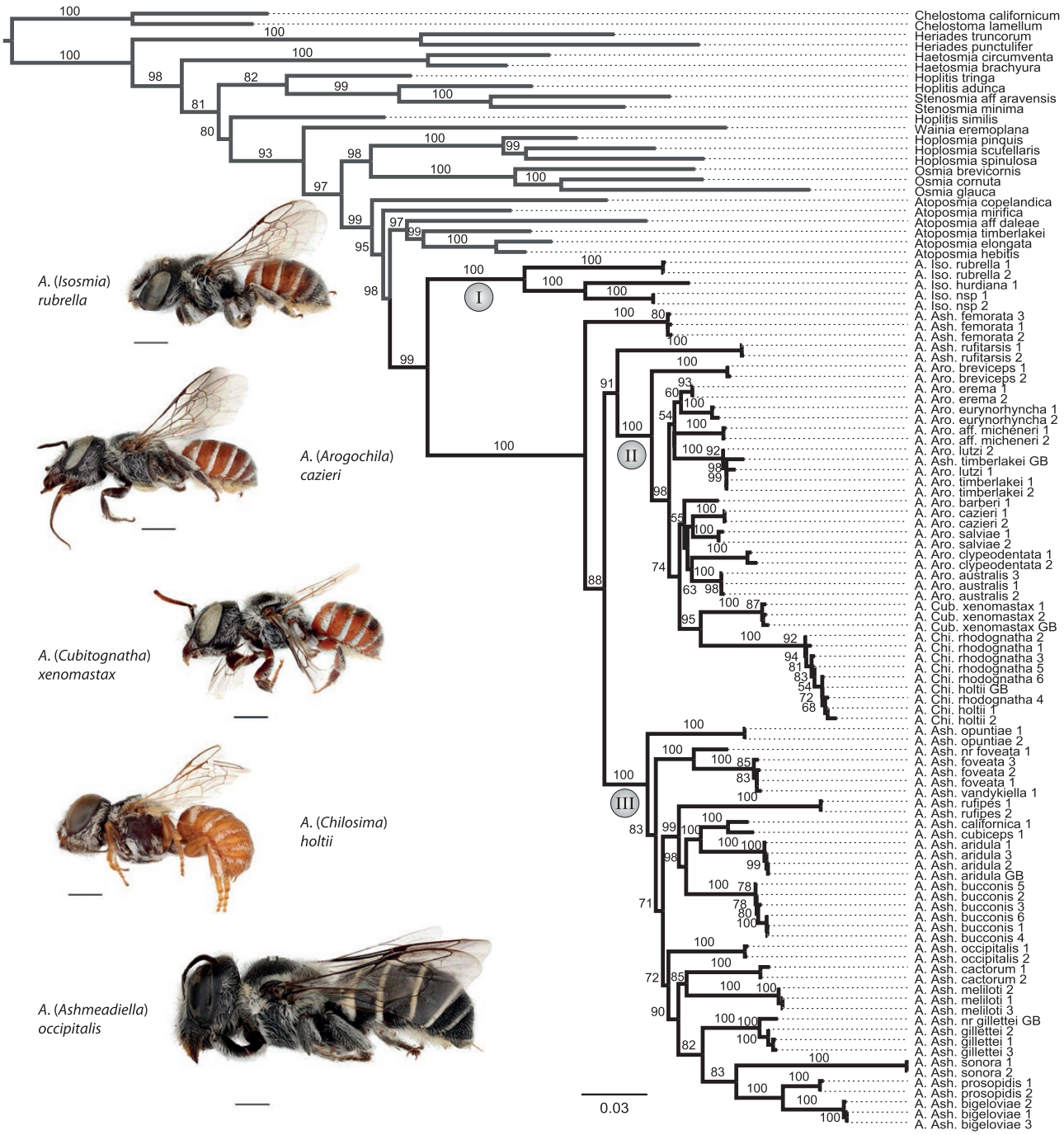

Fig. 1. Phylogeny of *Ashmeadiella*. The phylogeny is a maximum likelihood analysis of three concatenated genes: COI, CAD, and OPS. Branch support is shown by ultrafast bootstrap values. Roman numerals indicate the three major clades of *Ashmeadiella*, which mostly align to subgeneric groupings. The 'GB' after *Ashmeadiella* tip names indicates those sequences were from GenBank. All non-*Ashmeadiella* genera are GenBank specimens. The images on the left show a representative from each of the five described subgenera. The position of *A. femorata* as sister to the majority of *Ashmeadiella* is only found in the concatenated dataset analyses and the opsin gene tree analysis; COI and CAD gene tree analyses produce a nested placement of this species (see Supp Figs. 1–3 [online only]).

recorded number of flower family associates were *A.* (*Ash.*) *cactorum* (Cockerell) [24 families, 73 genera], *A.* (*Ash.*) *meliloti* [23, 59], and *A. (Ash.) bucconis* (Say) [18, 67] (for full list, see Supp Table 3 [online only]). These data suggest *Ashmeadiella* species are utilizing a wide variety of flowers. Furthermore, more than 30 species have fewer than five foraging reports (Table 1).

Diversity

Based on the available GPS data for specimens identified to species, *Ashmeadiella* communities seem to be richest in the Mojave Basin and Range with 34 species, followed by the Sonoran Desert with 30 species, and the Central Basin and Range with 28 species (Fig. 4). Areas in the Central Plains and southeast have less diversity, with some ecoregions having only one species collected there, and many eastern ecoregions have no records of *Ashmeadiella*. A plot of collections data by species shows that there are some species which have a marked increase in collections numbers through the years (up to the latest records from 2016), but there are many species for which collections have shown little yearly increase or have not been collected in the past several decades (Supp Fig. 6 [online only]).

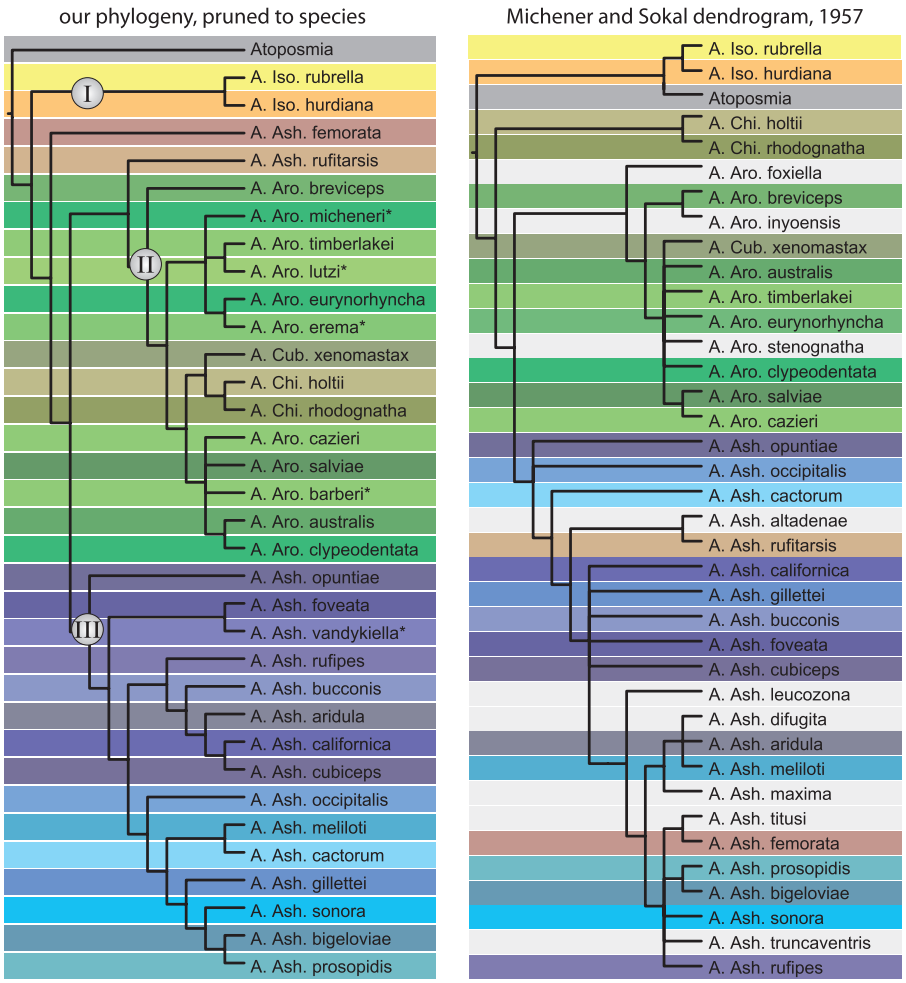

Fig. 2. Reduced phylogeny from Fig. 1 (left), and Michener and Sokal's (1957) dendrogram (right; modified from their Fig. 15) based on 122 characters in a pilot study of the numerical taxonomic method. Tip names follow current classification. The phylogeny from Fig. 1 has been reduced to one tip per species, and Roman numerals indicate the three major clades: Clade I) *Ashmeadiella* (*Isosmia*) [species colored in yellow and orange], Clade II) *A.* (*Arogochila*) + *A.* (*Chilosima*) + *A.* (*Cubitognatha*) [species colored in greens and browns], and Clade III) *Ashmeadiella s. str.* [species colored in blues and purples]. *Ashmeadiella* (*Ash.*) *femorata* and *A.* (*Ash.*) *rufitarsis* have differing placements in individual gene tree analyses and are not assigned clade membership. Asterisks indicate a species was not included in the Michener and Sokal dendrogram. The dendrogram at the right is colored according to corresponding species in our phylogeny, with no color for species that have no match in our dataset. Michener and Sokal (1957) treated *Atoposmia* and *Isosmia* as subgenera of *Anthocopa*.

Discussion

Phylogeny

Our molecular phylogeny provides a new hypothesis for *Ashmeadiella* species relationships and gives the first molecular evidence supporting subgeneric groupings within the genus (Figs. 1 and 2). Our results indicate that previous circumscription of subgenera using similarity of morphological characters did not lead to recognition of natural evolutionary groups in all cases. Michener and Sokal (1957) applied the first test of numerical taxonomy to this group of megachilid bees. We revisited Michener and Sokal's conclusions by comparing their results to our molecular phylogeny of the genus (Fig. 2). There are broad patterns of overlap in our molecular phylogeny and in their dendrogram built from morphological similarities. Excluding *Isosmia*, the monophyly of the remaining *Ashmeadiella* is uncontested and has been long-recognized using morphological characters, including all males presenting a four-toothed tergum 6, a characteristic not found in *Isosmia* (Michener 1939). Though not shown in our concatenated tree, the CAD phylogeny indicates a potential relationship of the subgenus *Isosmia* (Clade I) and the genus *Atoposmia*, as in the Michener and Sokal results. In Clade II, *Arogochila* has very strong support in all gene trees of being paraphyletic, due to the inclusion of two other subgenera. Our analyses show that the subgenera *Chilosima* (characterized by males having three-toothed mandibles and females having irregularly rounded clypeal margins) and *Cubitognatha* (characterized by females having unique elbowed, bidentate mandibles) both render *Arogochila* nonmonophyletic. Michener and Sokal (1957) concluded *Chilosima* was sister to the rest of the genus *Ashmeadiella*, whereas we find it nested in the subgenus *Arogochila* in concatenated results. The largest subgenus, *Ashmeadiella*, was supported in the historical analysis and in our molecular analyses for the most part; though in our concatenated phylogeny, two species (*A. femorata* and *A. rufitarsis*) from the subgenus *Ashmeadiella s. str.* do not cluster with the rest of Clade III.

Taxonomic Implications

We propose that the subgenera *Chilosima* and *Cubitognatha* be synonymized with the subgenus *Arogochila*. This change affects three species: *A. rhodognatha* and *A. holtii*, currently in *Chilosima*, and *A. xenomastax*, currently in *Cubitogantha*. The placement of *Cubitognatha* within *Arogochila* was recognized decades ago (Fig. 2,

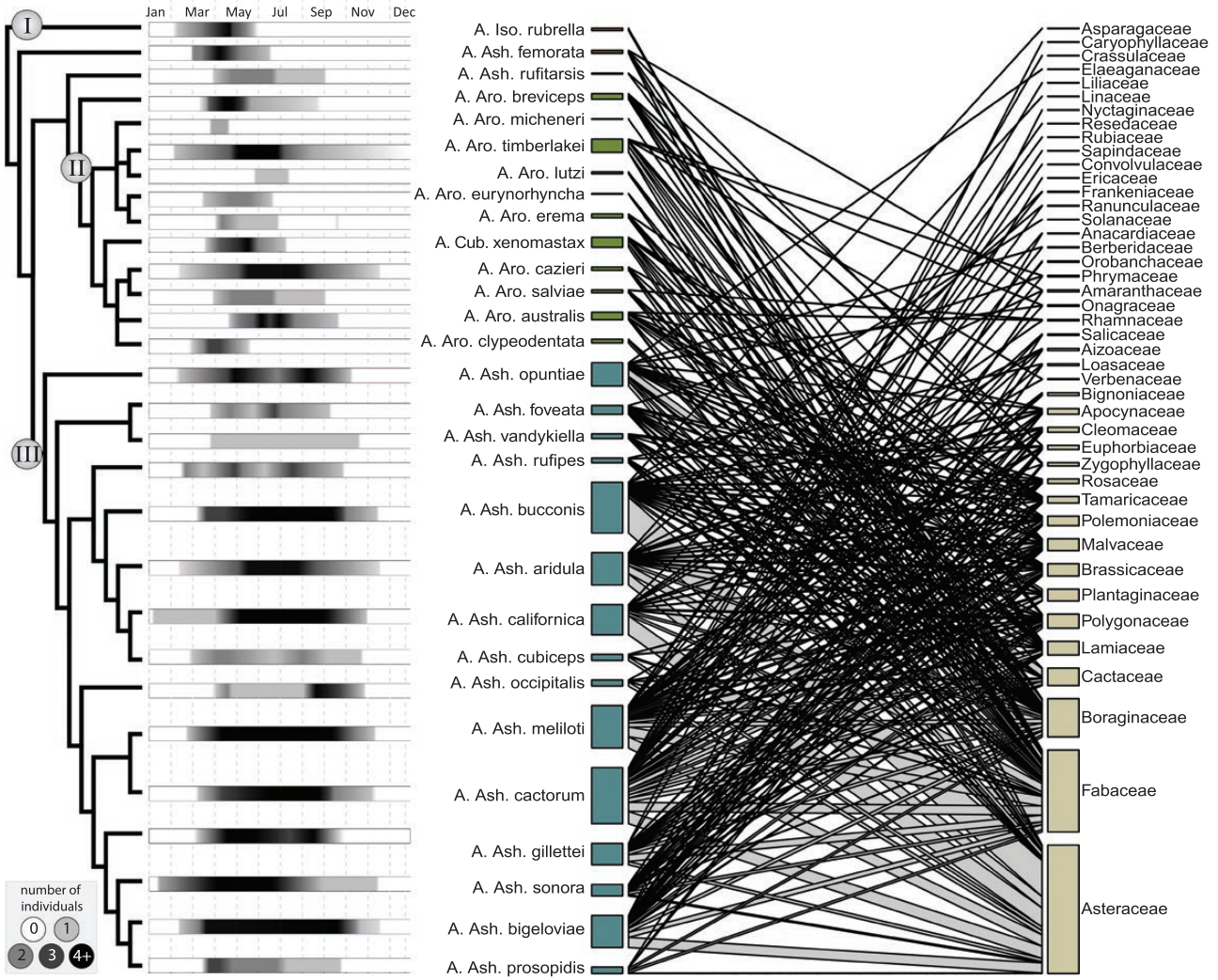

Fig. 3. Flight periods and bipartite network showing bee–host plant associations, visualized from specimen information of museum collections data. All bee records are ordered according to the *Ashmeadiella* phylogeny on the left. In the center are distributions of seasonality of databased bee records. On the right is the bipartite network. This network shows all *Ashmeadiella* species with flower data (male and female data were not separated; sex was rarely reported) on the left, and all host plant records at the family level on the right. Bee species boxes are colored by clade: orange = Clade I: subgenus *Isosmia,* green = Clade II: subgenera *Arogochila, Chilosima,* and *Cubitognatha,* and blue = Clade III: subgenus *Ashmeadiella.* Thickness of connection links between bees and host plant families denote the number of association records between them. See Supp Figs. 4 and 5 [online only] for complete records.

Michener and Sokal 1957), but no classification changes were proposed due to its morphological uniqueness.

The majority of species in the genus *Ashmeadiella* were recovered as monophyletic in our analyses, but this needs to be verified with additional sampling from across the geographic ranges of the species. In the three instances of nonmonophyletic pairs of species, the two species typically share morphological characters, but their biological data do not completely overlap. In the case of the clade of *A. lutzi* + *A. timberlakei*, it was previously noted that these two were very similar and one may be a subspecies of the other (Hurd and Michener 1955). Our analysis comparing adult flight periods show that *A. lutzi* has a short flight season, with 41 collection events on seven unique dates in the middle of the much longer flight season of *A. timberlakei* with 207 unique collection dates (Fig. 3, Table 1, Supp Fig. 4 [online only]), and *A. lutzi* has only been collected above latitude 35 (Supp Fig. 5 [online only]), which may be leading to temporal and geographical isolation. These differences in adult flight periods are likely not a lack-of-data error as both species have more than 30 different collection dates (Table 1). Furthermore, *A. lutzi* has been collected from four host genera in two families, with only two host genera overlapping with *A. timberlakei* (Supp Table 3 [online only]), which is possibly from a lack of data or a sign of early behavioral isolation between these two groups.

Our molecular data also suggest *A. vandykiella* and *A. foveata* are not distinct species. *Ashmeadiella vandykiella* is morphologically differentiated from *A. foveata* by three main characteristics: narrower cheeks (genal area), the presence of a pair of hairy spots at the anterior end of the mesoscutum, and the lack of a white pubescent band on T5, but Michener warned these two may key out similarly (Michener 1949). Our analyses also show flight times overlap, but *A. vandykiella* has a longer flight season and both species have similar latitudinal distribution (Supp Fig. 5 [online only]). Furthermore, floral records show that specimens have been collected on only four of the same plant species, with *A. foveata* reported from 19 species from 18 genera and ten plant families while *A. vandykiella* is reported from ten species, nine genera from seven plant families (Supp Table 3 [online only]); further research may show these species do forage on the same plants or may identify potential behavior leading to future sympatric speciation.

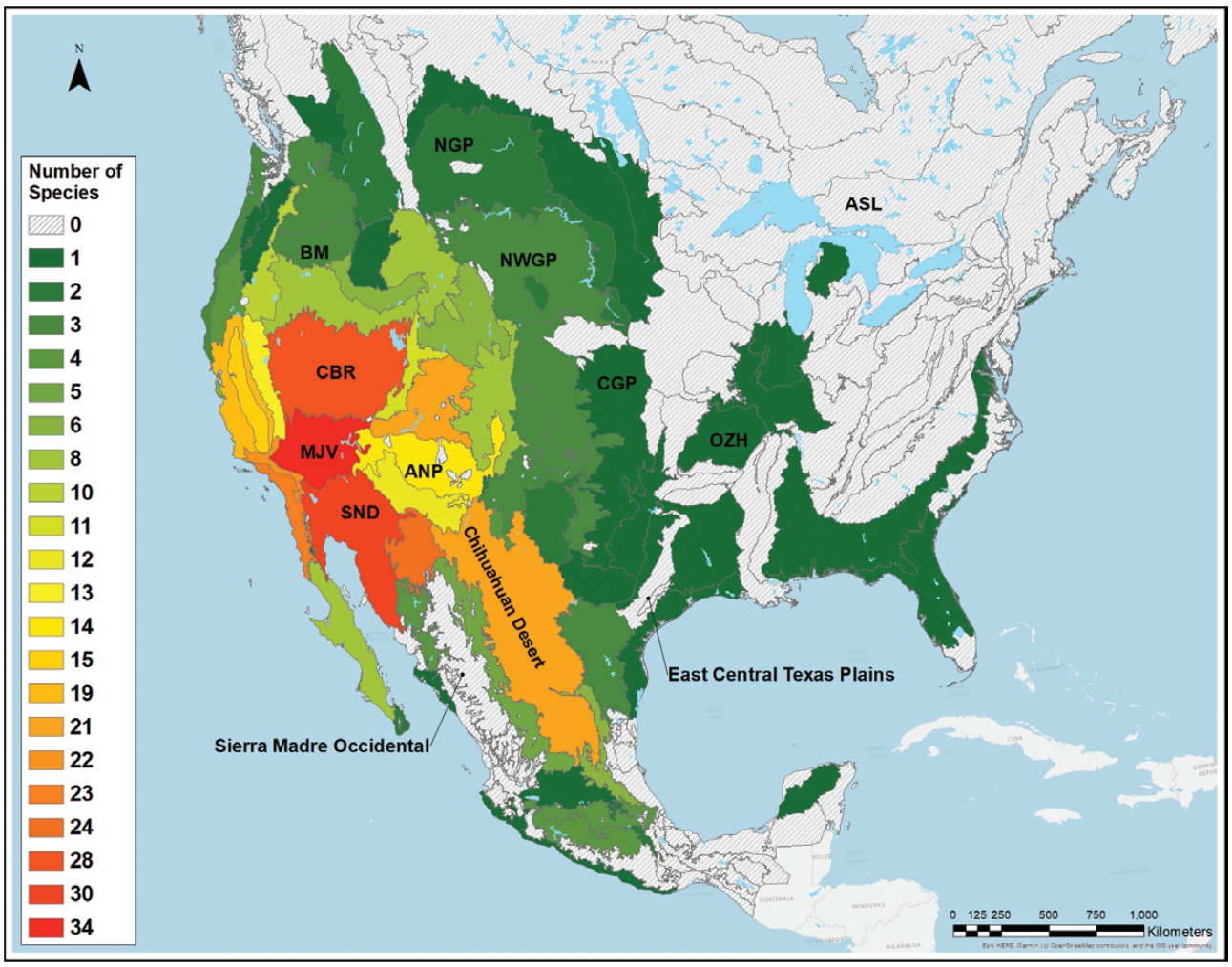

Fig. 4. Species richness of *Ashmeadiella* for Level III Ecoregions of North America (EPA 2020). *Ashmeadiella* communities appear to be most rich in the Mojave Basin and Range (MJV) which has also been heavily sampled with 34 species collected there, followed by the Sonoran Desert (SND) with 30 species, and the Central Basin and Range (CBR) with 28 species. Some ecoregions show no *Ashmeadiella* species, which may be correct for areas around the Great Lakes like the Algonquin/Southern Laurentians (ASL). Ecoregions such as the Arizona/New Mexico Mountains, which rise up from the Arizona/New Mexico Plateau (ANP), and Sierra Madre Occidental regions—among many others—likely need more sampling to document which *Ashmeadiella* species are present, as nearby ecoregions have documented *Ashmeadiella* species. Complete list of Ecoregion Level III names available through United States EPA (EPA 2020).

Based on the genes we used in this study, *A.* (*Chi.*) *rhodognatha* and *A.* (*Chi.*) *holtii* are not genetically distinct in either their phylogenetic relationships or COI distances. Michener differentiated *A. holtii* from *A. rhodognatha* based on coloration and described *A. holtii* based on a single male specimen (1939). Adults of *A. holtii* emerge around the same time as *A. rhodognatha* but stop flying much earlier. In addition, *A. holtii* has a more restricted latitudinal range, which may be signaling speciation via temporal and geographic isolation. Morphological differences may be caused by phenotypic or environmental parameters, or potentially there has been inadequate sampling to detect variation. There are currently no published floral records for *A. holtii*, so we cannot compare their diets; however, under the assumption of phylogenetic conservatism of pollen preference (Sedivy et al. 2008 and references therein), it is likely that they forage on the same flowers as *A. rhodognatha*.

Phenology, Forage, and Diversity

Although *Ashmeadiella* have been collected starting in the late 1800s, there is no research on individual species' total range, annual abundance, or habitat requirements, which would help establish if a species should be considered for listing under the Endangered Species Act. There are a few species that have not been reported since the turn of the century (Supp Fig. 6 [online only]) which follows a concerning broader pattern, as nearly a quarter of bee species globally have not been collected in nearly three decades (Zattara and Aizen 2021). Future research should target species that have not been recorded in the past decade as they may genuinely be in decline.

The available spatial data for *Ashmeadiella* suggests their communities are richest in the Mojave Basin and Range, followed by the Sonoran Desert and then the Central Basin and Range (Fig. 4). This pattern may change as more species are described and collected in less studied areas. Overall, there is a general trend of decreasing richness to the east and north. Some ecoregions show no *Ashmeadiella* species, which may be true for areas around the Great Lakes and eastern Canada. However, many collections are not yet digitally available, so some institutions likely have unpublished *Ashmeadiella* records. For example, *A. bucconis* is reported from Wisconsin (Mitchell 1962) but we could not find digital records verifying this report. In several ecoregions, more sampling is needed to document which *Ashmeadiella* species are present. Primary examples

include the Arizona/New Mexico Mountains, which rise up from the Arizona/New Mexico Plateau, areas south of the Ozark/Ouchita-Applachian Forests, and the Sierra Madre Occidental regions. These localities stand out because there are very few to no species reported within, yet they are surrounded by well sampled ecoregions that have diverse *Ashmeadiella* communities.

Using the available data for flight periods and floral associations, we visualized the biological information in a phylogenetic framework (Fig. 3). The species records for flight periods were grouped by clade, and there were marked differences in the overall duration of each clade but not in the overall emergence. Clade I (*Isosmia*) has one of the latest-emerging species, and all three described species have flight periods spanning less than 9 mo. Clade II (*Arogochila*) mostly have shorter flight periods that start in late spring and end before October. *Ashmeadiella (Aro.) neomexicana*, which was not included in our phylogeny so we cannot confidently place it in a clade, has an almost 11-mo flight period (Supp Fig. 4 [online only]). Clade III (*Ashmeadiella*) has four species with flight periods longer than 10 mo (Fig. 3). These data illustrate that conservation plantings should provide floral resources throughout portions of the year with warm days. Our synthesis of floral associations can be used for appropriate plant species to ensure floral resources are available during active flight seasons. Furthermore, our correlation analyses of latitude and emergence dates suggest some species are responding to temperature for emergence cues. Conversely, species where we do not see a correlation of emergence with latitude (Supp Fig. 5 [online only]) may be responding to other environmental cues such as precipitation.

Ashmeadiella are currently experiencing habitat loss due to energy development and urban expansion (McCoshum and Geber 2020) in the same ecoregions with the highest documented *Ashmeadiella* species diversity. Further surveys and collections are recommended because of the documented decline of bees and insects around the world (Colla et al. 2006, Kluser and Peduzzi 2007, Potts et al. 2010, Cameron et al. 2011, Hallmann et al. 2017, McArt et al. 2017, Cardoso and Gonçalves 2018, Mathiasson et al. 2019), hypothesized to be due to landscape-level stressors such as pathogens, pesticides, loss of habitat, and lack of flowers (Goulson et al. 2015, McArt et al. 2017). Currently, there is not enough baseline data for most *Ashmeadiella*, nor for many bee groups in the United States, for making clear distinctions of species' taxonomy, phylogenetic relationships, or life history. More collecting and observations of floral and pollen use will be needed to improve development of plans to monitor and conserve this group of charismatic pollinators.

Supplementary Data

Supplementary data are available at *Insect Systematics and Diversity* online.

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Author Contributions

EAM: Conceptualization, Data Curation, Formal Analyses, Investigation, Methodology, Validation, Visualization, Writing — Original Draft, Writing — review & editing. LE: Conceptualization, Data Curation, Formal Analyses, Investigation, Methodology, Resources, Validation, Writing — review & editing. SB Data Curation, Methodology, Writing — review & editing. MAG Conceptualization, Funding Acquisition, Methodology, Supervision, Writing — review & editing. TG: Conceptualization, Data Curation, Formal Analyses, Methodology, Resources, Validation, Writing — review & editing, SMM Conceptualization, Data Curation, Formal Analyses, Investigation, Methodology, Project Administration, Resources, Supervision, Validation, Writing — Original Draft, Writing — review & editing.

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