

RESEARCH ARTICLE

Young evolutionary origins of dioecy in the genus *Asparagus*

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Abstract

Premise: Dioecy (separate sexes) has independently evolved numerous times across the angiosperm phylogeny and is recently derived in many lineages. However, our understanding is limited regarding the evolutionary mechanisms that drive the origins of dioecy in plants. The recent and repeated evolution of dioecy across angiosperms offers an opportunity to make strong inferences about the ecological, developmental, and molecular factors influencing the evolution of dioecy, and thus sex chromosomes. The genus *Asparagus* (Asparagaceae) is an emerging model taxon for studying dioecy and sex chromosome evolution, yet estimates for the age and origin of dioecy in the genus are lacking.

Methods: We use plastome sequences and fossil time calibrations in phylogenetic analyses to investigate the age and origin of dioecy in the genus *Asparagus*. We also review the diversity of sexual systems present across the genus to address contradicting reports in the literature.

Results: We estimate that dioecy evolved once or twice approximately 2.78–3.78 million years ago in *Asparagus*, of which roughly 27% of the species are dioecious and the remaining are hermaphroditic with monoclinal flowers.

Conclusions: Our findings support previous work implicating a young age and the possibility of two origins of dioecy in *Asparagus*, which appear to be associated with rapid radiations and range expansion out of Africa. Lastly, we speculate that paleoclimatic oscillations throughout northern Africa may have helped set the stage for the origin(s) of dioecy in *Asparagus* approximately 2.78–3.78 million years ago.

KEYWORDS

Asparagaceae, Asparagoideae, chloroplast evolution, dioecy, divergence time estimation, fossil time calibration, phylogeny, plastome, polytomy

Most flowering plant species produce monoclinal flowers—stamens and pistils within the same flower—functioning as hermaphroditic individuals. However, approximately 6% of angiosperm species evolved separate sexes (dioecy) producing strictly pistillate or staminate flowers, functioning as either female or male individuals, respectively (Renner, 2014). Interestingly, dioecy has independently evolved numerous times in ancestrally hermaphroditic clades across the angiosperm phylogeny

(Ming et al., 2011; Carey et al., 2022). Theory suggests that dioecy is adaptive in that it precludes inbreeding through self-pollination (Charlesworth and Charlesworth, 1978; Thomson and Barrett, 1981) and can improve fertility through focused resource allocation for production of pollen (males) or ovules and seeds (females) (Darwin, 1877; Barrett, 1992; Ramsey et al., 2006). Both factors may contribute to the origins of dioecy in small populations occupying resource-limited environments.

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The two most prominent theories for the evolutionary transition from hermaphroditism to dioecy predict that dioecy evolves via (1) selection for distinct male and female genotypes in a monoecious population wherein the developmental pathway for unisexual flowers should already exist (Lewis, 1942; Renner and Ricklefs, 1995; Boualem et al., 2015) or (2) the origin of a female-sterilizing mutation in a gynodioecious (i.e., female and hermaphroditic individuals) population wherein a male-sterilizing mutation is already established (Darwin, 1877; Charlesworth and Charlesworth, 1978). It is also theoretically possible for dioecy to arise via androdioecy (i.e., population with male and hermaphroditic individuals) (Charlesworth and Charlesworth, 1978). However, androdioecy is rare in nature since males are less likely to invade hermaphroditic populations, due to the inherent pollen competition between selfing bisexual flowers and unisexual staminate flowers (Lloyd, 1975). Nonetheless, our understanding of the pathways to dioecy is limited for flowering plants and across the tree of life (Renner and Ricklefs, 1995; Abbott et al., 2017). The recent and repeated evolution of dioecy in flowering plants offers an opportunity to make strong inferences about the ecological, developmental, and molecular mechanisms influencing the early stages of dioecy and sex chromosome evolution (Charlesworth, 2019; Renner and Müller, 2021).

The genus *Asparagus* Tourn. ex L. (Asparagaceae) is an ideal system for studying the evolution of dioecy because it encompasses a mixture of hermaphroditic (exhibiting monoclinal flowers) and dioecious lineages (e.g., garden asparagus [*Asparagus officinalis* L.]) and dioecy is thought to have evolved recently in the genus (Galli et al., 1993; Norup et al., 2015; Harkess et al., 2016). The age and origin of dioecy in *Asparagus* should directly coincide with the evolution of sex chromosomes because dioecious phenotypes in *A. officinalis* and close relatives are controlled by the presence/absence of a 1-Mb segment on the Y chromosome (Harkess et al., 2017, 2020). *Asparagus* (215 species) is one of two genera in the Asparagaceae subfamily Asparagoideae, along with *Hemiphyllacus* S. Watson (5 species) (Pires et al., 2006; Chase et al., 2009; Seberg et al., 2012). The biodiversity hotspot for *Asparagus* is southern Africa, but the genus is widespread across temperate, semiarid, and tropical regions of Africa, Europe, and Asia (POWO, 2023). Interestingly, the sister genus, *Hemiphyllacus*, is endemic to Mexico and mostly grows in limestone or rhyolitic areas (Hernandez, 1995). Whereas *Asparagus* species exhibit a variety of aboveground forms that are upright, self-supporting/sprawling, or climbing, but are characterized by their non-photosynthetic modified leaves that are reduced to bracts apically and spurs or spines basally (Obermeyer et al., 1992), and cladodes (or phylloclades), which are modified axillary shoots that function as leaves (Nakayama et al., 2012) (Figure 1), all species of *Hemiphyllacus* exhibit true leaves that form a rosette growth habit (e.g., Figure 10) (Hernandez, 1995). Like most Asparagaceae lineages, both Asparagoideae genera produce storage roots (e.g., Figure 1K) with a wide array of

interspecific morphological diversity. Most *Asparagus* and all *Hemiphyllacus* species are hermaphroditic with strictly monoclinal flowers (Hernandez, 1995; Kanno and Yokoyama, 2011), which is the hypothesized ancestral state of Asparagoideae and *Asparagus* (Obermeyer, 1983; Fukuda et al., 2005; Norup et al., 2015). However, a minority of *Asparagus* species exhibit sexual dimorphism and are almost always described as dioecious with unisexual flowers (Kanno and Yokoyama, 2011), though contradicting claims have been made of monoecy (Renner and Müller, 2021) and gynodioecy (Norup et al., 2015) in *Asparagus*. To resolve such discrepancies in the literature and better understand how dioecy evolved in *Asparagus*, a comprehensive review of the total diversity of sexual systems present in the genus is required.

Phylogenetic analysis of three plastid genome (plastome) loci (*trnH-psbA*, *trnD-trnT*, and *3'ndhF*) and one nuclear gene (phytochrome C) suggests that *Asparagus* species cluster into six major clades (with varying bootstrap support): *Asparagus* (89%), *Racemose* (65%), *Lignosus* (71%), *Africani-Capenses* (93%), *Myrsiphyllum* (81%), and *Setaceus* (67%) (Norup et al., 2015). Although relationships amongst these clades were weakly supported (all <65% bootstrap support), Norup et al. (2015) discussed morphological evidence and past taxonomical work supporting their veracity. Nonetheless, the weakly supported inferences asserted by Norup et al. (2015) need to be tested through phylogenomic analyses. Of particular relevance for the utility of *Asparagus* as a model for the evolution of dioecy and sex chromosomes, published phylogenetic and biogeographic analyses have suggested that the genus arose and radiated in southern Africa, and dioecy evolved as ancestral species dispersed into northern Africa, Europe, and Asia (Fukuda et al., 2005; Kubota et al., 2012; Norup et al., 2015). Most extant dioecious species are distributed in the Mediterranean Basin and across Eurasia, while most hermaphroditic species occur in Sub-Saharan Africa, thus transitions from hermaphroditism to sexual dimorphism in *Asparagus* appear to be associated with long-distance dispersal out of southern Africa (Norup et al., 2015). This geographic distribution supports predictions that dioecy may evolve in response to selection for reduced inbreeding in small populations founded through long-distance dispersal and colonization of new habitats (Charlesworth and Charlesworth, 1978; Thomson and Barrett, 1981). Additionally, previous work suggests that dioecy may have either (1) independently evolved twice in *Asparagus*, once in a clade with *A. officinalis* and again in a clade with *Asparagus horridus* L. or (2) only once followed by a loss in at least one hermaphroditic lineage including *Asparagus pauli-guilelmi* Solms [syn. *Asparagus flagellaris* (Kunth) Baker] and *Asparagus petersianus* Kunth (Norup et al., 2015). However, support for nesting these hermaphroditic lineages within an otherwise dioecious clade was generally low (Norup et al., 2015). Results from a different analysis of five noncoding plastome loci (*rpl32*-



FIGURE 1 Photographs illustrating morphological diversity found in the Asparagoideae genera *Asparagus* and *Hemiphyllacus*. (A) *Asparagus acutifolius* showing staminate flowers with rudimentary pistil development and glabrous, spiny cladodes. (B) *A. cochinchinensis* with staminate flowers and flattened cladodes. (C) *A. officinalis* with red berries and subterete cladodes. (D) *A. asparagoides* showing bisexual, axillary flowers, and broad, leaf-like cladodes. (E) *A. alopercurus* showing bisexual flowers and hairy cladodes. (F, K, L) *A. exuvialis* bisexual (F) flower, (K) tuberous roots, and (L) peeling stem. (G, H) Contrasting growth habits between (G) the sprawling habit of *A. densiflorus* cv. Sprengerii, compared to (H) the climbing habit of *A. aethiopicus*. (I) *A. horridus* extremely spiny, glaucous cladodes and dried staminate flowers. (J) *A. falcatus* exhibiting bisexual flowers and broader, more leaf-like cladodes. (M, N) Lower stem spine morphology differences between (M) *A. aethiopicus* and (N) *A. densiflorus*. (O) Rosette-forming growth habit and true leaves of *Hemiphyllacus hintoniorum*. Image credits: (A) Philip C. Bentz. (B) Shin-Ming Ku (<https://www.inaturalist.org/observations/109812909>). (C) iNaturalist user Igorpysh (legal name unavailable) (<https://www.inaturalist.org/observations/150112770>). (D–H, J–N) John and Sandra Burrows. (I) Mason McNair. (O) Joey Santore (<https://www.inaturalist.org/observations/110933573>). iNaturalist photographs (licensed under <http://creativecommons.org/licenses/by-nc/4.0/>) were cropped to fit the figure with no other adjustments.

trnL, *trnQ*-5'*rps16*, *ndhF*-*rpl32*, *psbD*-*trnT*, and 3'*rps16*-5'*trnK*) weakly suggests a single origin of dioecy in the genus (Kubota et al., 2012), though none of the hermaphroditic taxa nested in the putative dioecious clade from Norup et al. (2015) were included in that analysis. To elucidate the number of putatively

independent origins of dioecy in *Asparagus*, a significant increase of sequence data may be required for phylogenomic analysis of strategically chosen taxa.

In a separate analysis based on four plastid genes (*matK*, *rbcL*, *atpB*, and *ndhF*), the stem age of *Asparagus* was estimated at 8.6–25 million years ago (Ma) (Chen

et al., 2013). This analysis included one hermaphroditic and three dioecious species of *Asparagus*, which allowed for inference of the age of dioecy in the genus (Renner and Müller, 2021), though the main objective of the Chen et al. (2013) study was to date the origins of family-level clades across Asparagales. Molecular dating experiments may be sensitive to undersampling of the focal clade, in extreme cases (i.e., when <10% of species are sampled) exacerbating age underestimation (Linder et al., 2005). Additionally, *A. officinalis* and other branches/clades crucial for investigating dioecy evolution in *Asparagus* were missing in analyses from Chen et al. (2013) (e.g., *A. horridus*, *A. flagellaris*, *A. petersianus*, and close relatives) and the resulting topology is incongruent with that of *Asparagus*-centered analyses (e.g., Kubota et al., 2012; Norup et al., 2015). Investigation of dioecy in the genus *Asparagus* requires a robust phylogenetic framework with increased sampling of dioecious lineages.

In this study, we investigate the diversity of sexual systems in *Asparagus* by comprehensively reviewing the literature and explore the evolution of dioecy in the genus through plastome-based phylogenomic analysis. Plastid gene and genome sequences are widely used to investigate species diversification (e.g., Steele et al., 2012; Ross et al., 2016) and divergence time estimates across angiosperms (e.g., Chen et al., 2013; McKain et al., 2016; Givnish et al., 2018) because (1) they are thought to evolve relatively clocklike (i.e., low to moderate evolutionary rate), (2) are conserved across the majority of flowering plant lineages, and (3) do not typically recombine in a way that is phylogenetically relevant (Doyle, 2022). We assembled 38 complete plastomes for strategically targeted species of *Asparagus* to generate a plastome phylogeny with representatives from all six major clades in the genus, including focused sampling of taxa pertinent to the investigation of dioecy and sex chromosome evolution. We then use the resulting phylogenomic inference to investigate the origin(s) of dioecy in *Asparagus* and apply fossil time calibrations from across the monocots to estimate the evolutionary age of dioecy in the genus. *Asparagus* has emerged as a model system for studying the evolution of dioecy and sex chromosomes in plants and has already contributed to our broad understanding of molecular dynamics related to the dioecy evolution in angiosperms (Charlesworth, 2019; Andreuzza, 2020; Harkess et al., 2016, 2017, 2020; Carey et al., 2021; Renner and Müller, 2021). The phylogeny and divergence time estimations we report in this study are foundational for future investigation of dioecy and sex chromosome evolution across the genus *Asparagus*.

MATERIALS AND METHODS

Review of sexual systems in *Asparagus*

Sexual systems for all species were scored based on a review of the literature and original species descriptions (e.g., Barker-Webb et al., 1836; Hooker, 1894; Baker, 1875, 1898;

Wildeman, 1913; Engler, 1922; Hutchinson and Dalziel, 1954; Andrews, 1956; Ōi, 1965; Valdés, 1980; Mill and Tan, 1984; Obermeyer, 1983, 1984; Rechinger, 1964, 1990; Obermeyer et al., 1992; Malcomber and Demissew, 1993; Zhengyi et al., 2000; Thulin, 2002; Demissew, 1995, 2008; Norup et al., 2015; Boubetra et al., 2017; Regalado et al., 2017; Kottaimuthu et al., 2019) and personal observations in the field. Species with diclinous flowers of only one sex type per plant (i.e., male or female) were scored as dioecious, while species bearing solely monoclinal flowers were scored as hermaphroditic. We also reviewed the literature on *Asparagus* for all other possible sexual systems (e.g., gynodioecy, androdioecy, and monoecy) as defined by Renner (2014). If there was no documentation of mating types for a species, then they were scored as unknown. When reports of a species' sexual system conflicted previous reports, unless direct evidence was presented in the more recent study, the original species description took precedence.

Review of species geographical distributions

Biogeographical distributions were compiled for all species of *Asparagus* according to the International Plant Names Index (IPNI; <https://www.ipni.org>) using the Royal Botanic Gardens, Kew's Plants of the World Online (POWO, 2023). We defined species' geographical distribution according to Biodiversity Information Standards (<https://www.tdwg.org/standards/>), also known as Taxonomic Databases Working Group's (TDWG) World Geographical Scheme for Recording Plant Distributions Standard (Brummitt, 2001). Distributions were recorded at the second level of TDWG's recording scheme, which divides continents into biologically informative regions/subcontinents (Brummitt, 2001).

Taxon sampling for phylogenetic analysis

We focused sampling on the Asparagaceae subfamily Asparagoideae, including 42 accessions from *Asparagus* and one *Hemiphylacus alatostylus* L. Hern. The 42 *Asparagus* accessions represent 39 distinct species that were strategically selected to include at least two representative species from each of the six major clades described by Norup et al. (2015), encompassing the full geographic range and morphological diversity of the genus. The sample included 19 accessions representing 16 hermaphroditic species with monoclinal flowers and 23 dioecious species of *Asparagus*. Voucher information, along with additional details regarding clade, geography, and sexual system for each *Asparagus* accession used in this study can be found in Table 1. Some samples originated from DNAs or published sequences that were used in earlier studies (i.e., Kubota et al., 2012; Steele et al., 2012; Norup et al., 2015; Harkess et al., 2017; Li et al., 2019; Wong et al., 2022). After reviewing the voucher specimen for these samples, we identified a subset with questionable species identifications. As such, in this study we reclassified and

TABLE 1 Taxon and voucher information for samples included in this study. Geographic distributions correspond to the level 3 geographical codes of the Taxonomic Databases Working Group (TDWG) (Brummitt, 2001) as reported by POWO (2023). Clade distinctions refer to the six major clades of *Asparagus* as described by Norup et al. (2015). Taxa with a dioecious sexual system exhibit staminate or pistillate flowers on separate plants and hermaphroditic taxa exhibit solely monoclinal flowers.

Taxon	Herb.	Voucher	Clade	Geographic distribution	Sexual system
<i>Asparagus acutifolius</i> L.	GA	P.C. Bentz 1	Asparagus	Mediterranean Basin	Dioecious
<i>Asparagus aethiopicus</i> L.	GA	P.C. Bentz 2	Racemose	Cape Province to North-West Province, South Africa	Hermaphroditic
<i>Asparagus alopecurus</i> (Oberm.) Malcomber & Sebsebe	C	M.V. Norup 56	Myrsiphyllum	W Cape Province, South Africa	Hermaphroditic
<i>Asparagus angulofractus</i> Ilijin in V.L. Komarov (ed.)	KUN	08cs299	Eurasia	Central Asia to SW Xinjiang, China	Dioecious
<i>Asparagus asparagoides</i> (L.) Druce	Steele et al. (2012)	Steele et al. (2012)	Myrsiphyllum	S Ethiopia to S Africa	Hermaphroditic
<i>Asparagus brachyphyllus</i> Turcz.	KUN	Xianh0366	Asparagus	E European Russia to South Korea	Dioecious
<i>Asparagus breslerianus</i> Schult. & Schult.f. in J.J. Roemer & J.A. Schultes	KUN	Chens11652	Asparagus	Caucasus to Mongolia and W. Pakistan	Dioecious
<i>Asparagus cochinchinensis</i> (Lour.) Merr.	KUN	14CS8762	Asparagus	Japan to Indo-China and Philippines (N. Luzon)	Dioecious
<i>Asparagus cooperi</i> Baker	GA	P.C. Bentz 10	Africani-Capenses	Tropical & S Africa, Arabian Peninsula, W India	Hermaphroditic
<i>Asparagus dauricus</i> Fisch. ex Link	KUN	NMDB20170809125	Asparagus	SE Siberia to NE Korea and E. China	Dioecious
<i>Asparagus densiflorus</i> (Kunth) Baker cv. 'Sprengeri'	CUHK	Wong 109	Racemose	Mozambique (Inhaca Is.) to S Africa	Hermaphroditic
<i>Asparagus densiflorus</i> (Kunth) Baker cv. 'Myersii'	CUHK	Wong 092	Racemose	Mozambique (Inhaca Is.) to S Africa	Hermaphroditic
<i>Asparagus denudatus</i> (Kunth) Baker	GA	P.C. Bentz 7	Africani-Capenses	E tropical & S Africa	Hermaphroditic
<i>Asparagus exuvialis</i> Burch.	J	Burrows & Burrows 15913	Asparagus	S rtropical & S Africa	Hermaphroditic
<i>Asparagus falcatus</i> L.	GA	P.C. Bentz 43	Racemose	SW Ethiopia to S Africa, Arabian Peninsula, India, Sri Lanka	Hermaphroditic
<i>Asparagus filicinus</i> Buch.-Ham. ex D.Don	KUN	WH-2012-0316	Asparagus	Himalaya to central China	Dioecious
<i>Asparagus flagellaris</i> (Kunth) Baker	J	Burrows & Burrows 9708	Asparagus	Tropical & S Africa, W Arabian Peninsula	Hermaphroditic
<i>Asparagus gobicus</i> N.A. Ivanova ex Grubov	KUN	NMZA0256	Asparagus	Mongolia to N China	Dioecious
<i>Asparagus horridus</i> L.	GA	P.C. Bentz 32	Asparagus	Canary Is., Mediterranean Basin to Arabian Peninsula	Dioecious
<i>Asparagus kiusianus</i> Makino	TUS	TUS 549770	Asparagus	Japan (SW Honshu, N & W Kyushu)	Dioecious
<i>Asparagus laricinus</i> Burch.	GA	P.C. Bentz 27	Lignosus	Congo to Tanzania and S Africa	Hermaphroditic

(Continues)

TABLE 1 (Continued)

Taxon	Herb.	Voucher	Clade	Geographic distribution	Sexual system
<i>Asparagus longiflorus</i> Franch.	KUN	LiuJQ-GN-20111-177	Asparagus	Qinghai to N central China	Dioecious
<i>Asparagus lycopodiineus</i> (Baker) F.T. Wang & Tang	KUN	12CS4184	Asparagus	Bhutan to central China	Dioecious
<i>Asparagus meiolados</i> H.Lév.	KUN	ZhangDC-07ZX-2024	Asparagus	S central China	Dioecious
<i>Asparagus myriacanthus</i> F.T. Wang & S.C. Chen	KUN	SCSB-B-000454	Asparagus	SE Tibet to China (NW Yunnan)	Dioecious
<i>Asparagus neglectus</i> Kar. & Kir.	KUN	16CS13513	Asparagus	Siberia to W Himalaya	Dioecious
<i>Asparagus nelsii</i> Schinz	C	M.V. Norup 142	Graniticus	Zambia to S Africa	Hermaphroditic
<i>Asparagus officinalis</i> L.	KUN	SunY081	Asparagus	Europe to Mongolia	Dioecious
<i>Asparagus oligoclados</i> Maxim.	KUN	WangCh387	Asparagus	Mongolia to Japan (Kyushu)	Dioecious
<i>Asparagus</i> cf. <i>pseudoscaber</i> Grecescu	GA	P.C. Bentz 83	Asparagus	Romania, Ukraine, Yugoslavia	Dioecious
<i>Asparagus petersianus</i> Kunth	J	Burrows & Burrows 8852	Asparagus	Tanzania to Mozambique	Hermaphroditic
<i>Asparagus recurvispinus</i> (Oberm.) Fellingham & N.L. Mey.	J	Burrows & Burrows 9428	Africani-Capenses	Cape Province, South Africa	Hermaphroditic
<i>Asparagus retrofractus</i> L.	C	M.V. Norup 110	Lignosus	Namibia to Cape Province, South Africa	Hermaphroditic
<i>Asparagus schoberioides</i> Kunth	TUS	TUS 549769	Asparagus	SE Siberia to Japan	Dioecious
<i>Asparagus</i> cf. <i>setaceus</i> (Kunth) Jessop	J	Burrows & Burrows 7817	Setaceus	Central Ethiopia to S Africa, Comoros	Hermaphroditic
<i>Asparagus setaceus</i> (Kunth) Jessop	J.-R. Li et al. (2019)	J.-R. Li et al. (2019)	Setaceus	Central Ethiopia to S Africa, Comoros	Hermaphroditic
<i>Asparagus taliensis</i> F.T. Wang & Tang ex S.C. Chen	KUN	YNAU_Atta_H_01	Asparagus	China (Yunnan)	Dioecious
<i>Asparagus tenuifolius</i> Lam.	GA	P.C. Bentz 44	Asparagus	Mediterranean Basin to Ukraine	Dioecious
<i>Asparagus tibeticus</i> F.T. Wang & S.C. Chen	KUN	ZhongY130	Asparagus	Tibet	Dioecious
<i>Asparagus trichoclados</i> (F.T. Wang & Tang) F.T. Wang & S.C. Chen	KUN	GLGE12287	Asparagus	W Asia, Mongolia to N China	Dioecious
<i>Asparagus virgatus</i> Baker	GA	P.C. Bentz 15	Setaceus	Yemen, S Tanzania to S Africa	Hermaphroditic
<i>Asparagus virgatus</i> Baker	TUS	TUS 549771	Setaceus	Yemen, S Tanzania to S Africa	Hermaphroditic
<i>Hemiphylacus allostylus</i> L. Hern.	Steele et al. (2012)	Steele et al. (2012)	<i>Hemiphylacus</i>	Mexico	Hermaphroditic

Note: Herb. = Herbarium acronym in accordance with Index Herbariorum (<https://sweetgum.nybg.org/science/ih/>). Citations are listed in place of voucher information for samples obtained from NCBI's GenBank, except for accessions from Wong et al. (2022).

updated species names for the following voucher specimens from Norup et al. (2015): *M.V. Norup 56* (previously *Asparagus cf. juniperoides* Engl.) as *Asparagus alopecurus* (Oberm.) Malcomber & Sebsebe; *M.V. Norup 110* [previously *Asparagus cf. concinnus* (Baker) Kies] as *Asparagus retrofractus* L.; *Burrows & Burrows 9428* [previously *Asparagus* “karooicus” as *Asparagus recurvispinus* (Oberm.) Fellingham & N.L. Mey.]; and *Burrows & Burrows 7817* (previously *Asparagus* sp. indetermined) as *Asparagus cf. setaceus* (Kunth) Jessop. Further, we identified *K.H. Wong 109* (previously *Asparagus aethiopicus* L.) from Wong et al. (2022) as *Asparagus densiflorus* (Kunth) Baker cv. Sprengerii.

Taxon sampling was extended across Asparagales (59 total samples) including all other Asparagaceae subfamilies except for Aphyllanthoideae: Agavoideae (6 samples), Nolinoideae (2), Brodiaeoideae (1), Lomandroideae (1), Scilloideae (3). Aphyllanthoideae is a monotypic subfamily that, when included in phylogenetic analysis, tends to decrease support values of multiple neighboring branches (Graham et al., 2006; Givnish et al., 2018). Sampling was further extended across select monocot orders to include Acorales (1 sample), Alismatales (1), Poales (1), Zingiberales (2), Petrosaviales (1), Liliales (2), Arecales (1), and Pandanales (2) to provide nodes for fossil time calibrations in divergence time analyses since no reliable calibration time points exist for *Asparagus*. *Magnolia grandiflora* was included as an outgroup to the monocot clade. Additional sample details and source information can be found in Appendix S1.

DNA-sequencing data generation

Plastome sequences were extracted from either whole-genome sequencing (WGS) or mining off-target Hyb-Seq reads from experiments with Angiosperms353 (Johnson et al., 2018) or Asparagaceae1726—a recently developed probe set for Hyb-Seq and phylogenomics in Asparagaceae (P. C. Bentz and J. Leebens-Mack, University of Georgia, unpublished data). Mining off-target reads from target-enriched sequencing data is possible due to the high copy number of organellar genomes in sequencing data (Graham et al., 2006; Bratzel et al., 2023). In sum, DNA was isolated from silica-dried or flash-frozen leaf tissue using Quick-DNA Plant/Seed Miniprep Kit (Zymo Research, Irvine, CA, USA), DNeasy Plant Mini Kit (Qiagen, Nordic, Copenhagen, Denmark), or Biomarker Plant DNA Kit (Beijing Biomarker Biotechnology, Beijing, China) according to the manufacturer's protocol. DNA-sequencing (DNA-Seq) libraries were prepared with KAPA HyperPlus Kit (Roche, Basel, Switzerland) with custom adapters and iTru primers from Adapterama I (Glenn et al., 2019) or NEBNext Ultra II DNA PCR-free Library Prep Kit (New England Biolabs, Ipswich, MA, USA). Libraries used in Hyb-Seq experiments were pooled before hybridization reactions using standard manufacturer recommendations with myBaits Custom DNA-Seq Kit v.4. Libraries (Daicel Arbor Biosciences,

Ann Arbor, MI, USA), and pools were checked for quality using Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA) and quantified using real-time PCR (qPCR) with KAPA Library Quantification Kit and KAPA SYBR Fast qPCR Master Mix. DNA-Seq libraries were sequenced using Illumina NovaSeq. 6000, NextSeq. 500, or NextSeq. 2000 flow cells at the HudsonAlpha Institute for Biotechnology (Huntsville, AL, USA), Georgia Genomics and Bioinformatics Core (Athens, GA, USA), or Beijing Biomarker (Beijing, China). See Appendix S1 for DNA preparation details for each sample.

Plastome assembly and annotation

We removed sequencing adapters from the reads, corrected mismatched base pairs, and removed reads shorter than 21 bases using fastp v.0.23.2 (Chen et al., 2018). Plastid sequence assemblies were generated de novo using GetOrganelle v.1.7.5.2 (Jin et al., 2020) with default parameters for circular plastome assembly and annotated using a reference-based approach with PGA, which uses a reverse query-subject BLAST search approach to accurately identify gene and intron boundaries (Qu et al., 2019). We used PGA with default parameters and the following references obtained from GenBank: *Asparagus cochinchinensis* (NC_060472), *Asparagus filicinus* (NC_046783), *Asparagus setaceus* (NC_047458), *Zamia furfuracea* (JX416857), and *Amborella trichopoda* (AJ506156). *Zamia furfuracea* and *A. trichopoda* are default reference plastomes in PGA, since they contain some of the highest gene numbers across seed producing plants (Qu et al., 2019), and we added the *Asparagus* references to aid the BLAST homology search. All plastome sequence assemblies and annotations were visually inspected in Geneious Prime v.2021.2.2 (Biomatters, Auckland, New Zealand).

Gene sampling for phylogenetic analysis

Plastid gene sequences were compiled for a total of 70 monocots and the outgroup *M. grandiflora*. We used gene sequences from 77 protein-coding genes, rather than complete plastome sequences, to account for missing data across samples. Notably, *ycf1*, *ycf2*, *accD*, and *infA* were not included in phylogenetic analysis due to high sequence variability and alignment difficulty (Givnish et al., 2018; H.-T. Li et al., 2019). The complete list of sampled genes is in Appendix S2.

Phylogenetic analysis

We used MAFFT v.7.487 (Katoh and Standley, 2013) to produce separate multiple sequence alignments (MSAs) for each of the 77 plastome genes by employing MAFFT's L-INS-i alignment strategy, an iterative refinement approach that incorporates local pairwise alignment information and typically results in a more accurate alignment (Katoh and

Standley, 2013). Separate gene alignments were concatenated into a supermatrix using SequenceMatrix v.1.9 (Vaidya et al., 2011). The supermatrix was analyzed by maximum likelihood (ML) using IQ-TREE v.2.2.0 (Minh et al., 2020) with 1000 ultrafast bootstrap approximations (UFBoot) (Hoang et al., 2018) and again with 100 standard non-parametric bootstraps (SBS) for comparison. UFBoot is an IQ-TREE function that helps reduce the use of computational resources compared to SBS and still provides relatively unbiased branch support (Minh et al., 2013). We partitioned genes in the supermatrix and determined substitution models according to IQ-TREE's ModelFinder (Kalyanamoorthy et al., 2017), allowing each partition to evolve under its own evolutionary model (Chernomor et al., 2016). To remain consistent with downstream divergence time estimation analyses, we employed the TESTMERGE option in IQ-TREE, which instructs ModelFinder to only consider the invariable site and gamma rate heterogeneity models.

We then used Phycas v.2.2.0 to evaluate fully bifurcating trees and tree topologies with polytomies in a Bayesian framework (Lewis et al., 2015). The concatenated gene set used for Phycas was the same as that used in the ML analysis, but only Asparagoideae taxa were included. We compared results from two independent runs of Phycas (with different random number seeds) for each of the two search strategies: one that invokes a polytomy prior with the flag `mcmc.topo_prior_C = 1`, thus evaluating trees with polytomous branching, and another that did not invoke the polytomy prior, which directs the Markov chain Monte Carlo (MCMC) search to only evaluate the likelihoods of fully bifurcating trees. All Phycas runs used GTR+ Γ substitution models with four rate categories and allowed for invariable sites. The MCMC for each run was repeated for 100,000 cycles with a 10% burn-in, sampling every 100 cycles. Fewer MCMC cycles are needed in Phycas, compared to other Bayesian phylogenetic software, since Phycas attempts to update most parameters at least once every cycle (Phycas v.2.2.0; Lewis et al., 2015). Posterior probabilities were compared for each branch between the two Phycas search strategies (i.e., with and without assessment of topologies with polytomous branches) to assess whether bifurcating or polytomous branching patterns had greater support and whether well-supported polytomies might be a consequence of insufficient data or truly polytomous branching. Comparison of the two replicate runs for each Phycas search strategy was performed to test for convergence. Resulting trees from all ML and Phycas analyses were initially plotted using R v.4.2.2 (R Core Team, 2020) with a combination of the following packages: ape (Paradis and Schliep, 2018), treeio (Wang et al., 2019), phytools (Revell, 2012), ggplot2 (Wickham, 2016), and ggtree (Yu et al., 2017).

Divergence time estimation

Divergence times were estimated on all nine data partitions from the ML analysis using BEAST v.2.7.2

(Bouckaert et al., 2019) and BEAUti v.2.7.3 (Bouckaert et al., 2019) to setup the BEAST input XML file. We arbitrarily chose the UFBoot ML tree, rooted with the outgroup *M. grandiflora*, to constrain tree topology in BEAST, which restricted the MCMC to estimate divergence times and not sequence relationships. Tree topology constraints were applied in BEAUti by setting the weight of the operators Bacterian Subtree Slide, Exchange (Narrow), Exchange (Wide), and Wilson Balding to zero. Using BEAUti, we scaled the input branch lengths of the tree by a factor of 1100 $\times$, effectively scaling branches to units of time in millions of years.

As in the ML analysis, we allowed each partition to evolve separately in BEAST by unlinking site and clock models. However, trees were linked between partitions because plastomes do not recombine in a way that is phylogenetically significant (Doyle, 2022). Substitution rates and proportion of invariant sites were also estimated during the BEAST run. GTR+ Γ nucleotide substitution models with four gamma rate categories were applied to each partition. We used a Yule tree prior and estimated clock rates using an optimized relaxed clock (Douglas et al., 2021) for all partitions. An initial clock rate of 1e-3 was set, to help scale clock estimates (i.e., branch lengths) to million years ago. To constrain branching time estimations, we used age estimates for five monocot fossils based on confidence of fossil age/taxonomy (see review of these fossils by Iles et al., 2015) and availability of plastome sequences for clades with representative fossils. Fossil time calibrations were applied using a lognormal distribution with a mean of 1.0, standard deviation (SD) of 2.0, and a minimum age for the following nodes: offset (or min.) of 14.5 Ma (Perkins et al., 1998) for the crown group of Agavoideae (Asparagaceae) (Tidwell and Parker, 1990); offset of 22 Ma (Giret et al., 1989) for the crown group of Lomandroideae (Asparagaceae) (Conran, 1997); offset of 47 Ma (Franzen, 2005; Mertz and Renne, 2005) for the crown group of Cyclanthaceae (Smith et al., 2008); offset of 52 Ma (Carpenter et al., 2007) for the stem group of Ripogonaceae (Conran et al., 2009); and offset of 81.13 Ma (Hicks, 1993) for the stem group of Aponogetonaceae (Grímsson et al., 2014). To constrain total tree height, we also applied one secondary calibration with a normal distribution on the stem node of all included monocot taxa with a mean of 1.0, SD of 1.0, and offset of 133 Ma (Magallón et al., 2015; Givnish et al., 2015, 2018). Seven independent BEAST runs were implemented with different starting seeds, with the same data set and parameters. Resulting log and tree files were combined using LogCombiner v.2.7.1 (Bouckaert et al., 2019). After 10% burn-in, all BEAST runs were continued until effective sample sizes (ESS) were equal to or greater than 200 for each parameter. To reach an ESS > 200, all independent BEAST runs were combined for a total of 671,039,000 MCMC generations with sampling every 1000 generations. The MCMC ESS and convergence were assessed using Tracer v.1.7.2 (Rambaut et al., 2018). A maximum clade credibility tree with mean node heights using a sample of 477,185 post burn-in trees (after 60% burn-in), to avoid unnecessary usage

of computational resources, was produced using TreeAnnotator v.2.7.1 (Bouckaert et al., 2019).

Since much uncertainty exists when assessing correlation between fossil age and the age of the most recent common ancestor (MRCA) of a clade, best practice may be to apply vague prior densities (Barido-Sottani et al., 2018). To test whether prior densities had a significant effect on divergence time estimates, we replicated the above BEAST analysis, but instead of using a lognormal prior for each fossil calibration, we applied a uniform distribution using the offset value as the minimum age with no maximum. Using these priors, we again implemented seven independent BEAST runs and used the same tools and criteria for assessing MCMC ESS and convergence. All together, these supplemental BEAST runs summed to 674,345,000 MCMC generations (after 10% burn-in) and a resulting maximum clade credibility tree estimated using a sample of 472,043 trees (after 60% burn-in). The maximum clade credibility trees from each BEAST run were plotted and polished using the same methods as we did for the ML trees, along with the R package rBt (Sánchez-Ramírez, 2018) to plot the 95% highest posterior density (HPD) intervals.

RESULTS

Geographic distribution of monoclony and dicliny in *Asparagus*

Of the 215 accepted species of *Asparagus*, 58 (or 26.98%) were scored as dioecious with diclinous flowers, 142 (66.05%) as hermaphroditic with monoclinal flowers, and 15 (6.97%) with unknown sexual systems and flower types. We found no supporting evidence in the literature for gynodioecious, androdioecious, nor monoecious species of *Asparagus*. Of the 142 hermaphroditic species, 111 (78.2%) are native only to regions south of the Sahara Desert in Africa, including nine Madagascar endemics, and do not overlap geographically with dioecious species. However, a small number of hermaphroditic species overlap with dioecious species in the Mediterranean Basin of northern Africa (3 species, one of which extends into southern Europe [*Asparagus albus* L.]), Macaronesia (8), Arabian Peninsula (6), and India (19). In Macaronesia, all species but *Asparagus pastorianus* Webb & Berthel., which also occurs in the Mediterranean Basin, are endemic to the region. Of the Indian species, 15 are endemic, and the remaining four also occur in the Arabian Peninsula. Three hermaphroditic species from India have the widest geographic ranges in the genus: *Asparagus africanus* Lam., *Asparagus racemosus* Willd., and *Asparagus falcatus* L. Altogether, the range of these three species extends across the African continent (excluding north of the Sahara Desert), Arabian Peninsula, and India. *Asparagus racemosus* is the most widespread species in the genus, with a reported range extending from Africa to China and Australia.

Of the 58 dioecious species of *Asparagus*, 29 (50%) are native to China, 13 of which have broader ranges extending from eastern Europe to India, Siberia, and eastern Asia. Seven total dioecious species occur in eastern Asia, two of which are endemic to Japan (i.e., *Asparagus kiusianus* Makino and *Asparagus pygmaeus* Makino). Seven dioecious species extend into India, though none are endemic there. The remaining dioecious species are distributed across western Asia (10), middle Asia (5), and Europe (12). Of the European dioecious species, only one extends into northern Europe (i.e., *Asparagus prostratus* Dumort.), while three extend into Asia (i.e., *Asparagus verticillatus* L., *Asparagus inderiensis* Blume ex Ledeb., and *Asparagus tenuifolius* Lam.). *Asparagus tenuifolius* also occurs in the Mediterranean Basin along with three other taxa from Europe: *Asparagus horridus* L., *Asparagus acutifolius* L., and *Asparagus aphyllus* L. Unlike *A. tenuifolius*, these three species are concentrated in the Mediterranean Basin, inhabiting countries from all three continents that border the Mediterranean Sea (i.e., Africa, Asia, and Europe). *Asparagus horridus* is also reported in Macaronesia. A summary of species distributions with monoclinal versus diclinous flowers is illustrated in Figure 2. Additional details regarding taxa included in this study can be found in Table 1, and the full matrix for all 215 species of *Asparagus* is in Appendix S3.

Plastome assembly and annotation

Complete circularized plastome assemblies were generated for 37 of the 38 samples of *Asparagus* collected for this study. A complete plastome did not assemble from the Angiosperms353 off-target reads for *A. cf. setaceus* (Burrows & Burrows 7817); however, we were able to assemble 75 of the 77 genes used in phylogenetic analyses (Appendix S4). Plastome assemblies for the other 37 samples resulted in relatively similar lengths, ranging from 155,655 bases in *Asparagus angulofractus* Iljin (08cs299) to 157,164 bases in *Asparagus cooperi* Baker (P.C. Bentz 10). Overall, there were no major outliers amongst the 37 complete plastome assemblies, in terms of gene content, order, and organization, compared to the common angiosperm plastome (i.e., quadripartite structure with two copies of a large inverted repeat that are separated by one large and one small single-copy region) (Guisinger et al., 2010).

Phylogenetic analyses

According to Bayesian information criterion (BIC), the best-fit partition model for these data used a total of nine partitions, each using their own substitution model (Appendix S2). Branch support in the ML analyses was at least 98% for relationships among all included monocot taxonomic orders, except for the relationship between

Arecales, Poales, and Zingiberales (UFBoot = 65; SBS = 40) (Appendix S5, Figures S2, S4). The UFBoot and SBS were 100% for the family Asparagaceae as a monophyletic clade. Within the subfamily Asparagoideae, both ML analyses yielded 100% support for *Hemiphylacus* as sister to *Asparagus* in ML analyses and 100% bootstrap support for six major clades of *Asparagus* (Figure 3). Support for relationships among these clades were as follows: Setaceus clade sister to all other clades (UFBoot = 100; SBS = 100); Myrsiphyllum sister to Africani-Capenses clade (UFBoot = 84; SBS = 70); Myrsiphyllum and Africani-Capenses clade sister to Lignosus, Racemose, and Asparagus clade (UFBoot = 100; SBS = 100); Lignosus sister to Racemose clade (UFBoot = 95; SBS = 82); Lignosus and Racemose clade sister to Asparagus clade (UFBoot = 100; SBS = 100) (left panel in Figure 4). Further, ML analyses showed 100%

support for *Asparagus exuvialis* Burch. as sister to all other taxa in the *Asparagus* clade, including all dioecious and two hermaphroditic species (*A. petersianus* and *A. flagellaris*) (left panel in Figure 4). Support for a single dioecious clade was lacking in the SBS analysis (UFBoot = 86; SBS = 58) (left panel in Figure 4). However, there was 100% UFBoot and SBS support for monophyly of the Mediterranean Basin dioecious clade (*A. horridus* and *A. acutifolius*) and mixed support for monophyly of the Eurasia dioecious clade (UFBoot = 84; SBS = 89) (left panel in Figure 4). The remainder of phylogenetic relationships across the genus *Asparagus* resulted in mixed SBS/UFBoot support, ranging from 59% to 100% (left panel in Figure 4).

Both Phycas runs resulted in the same topology as the ML analyses, aside from the eight strongly supported polytomies (posterior probability [PP] = 1) in replicate

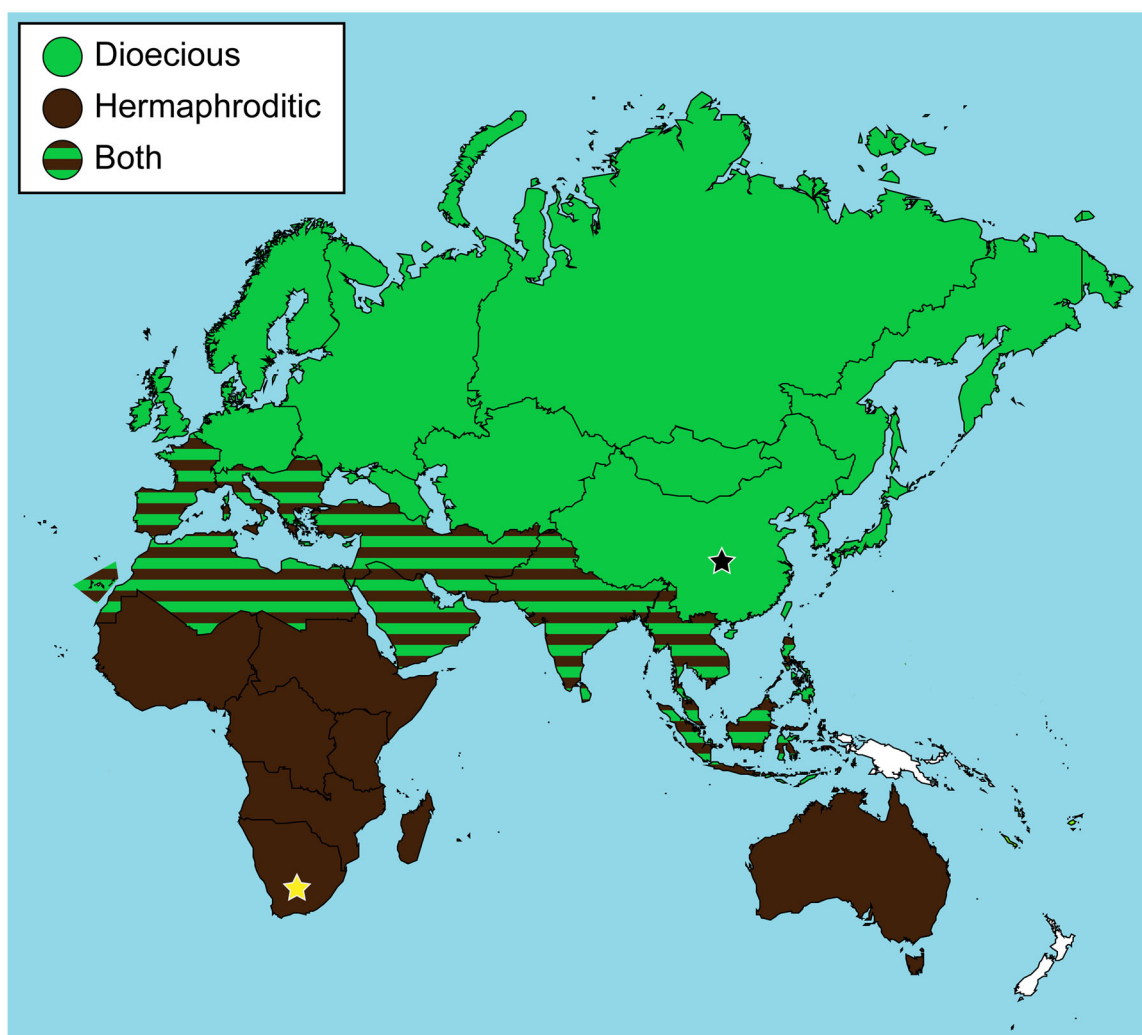


FIGURE 2 Extant biogeographic distribution of sexual systems in the genus *Asparagus*. Green = dioecious species with dichinous flowers; brown = hermaphroditic species with monoclinal flowers; striped = both dioecious and hermaphroditic species. Notably, the anomalously widespread *A. racemosus* is the sole species native to Australia (Batchelor and Scott, 2006). Species distributions illustrated here are based on the putative native range for all accepted species of *Asparagus* (POWO, 2023) and geographical borders correspond to Taxonomic Databases Working Group's second level of classification (Brummitt, 2001). Yellow star in southern Africa is the major radiation center and ancestral range of *Asparagus*, while the black star in southeastern Asia represents a secondary radiation hotspot composed of dioecious species (Norup et al., 2015).

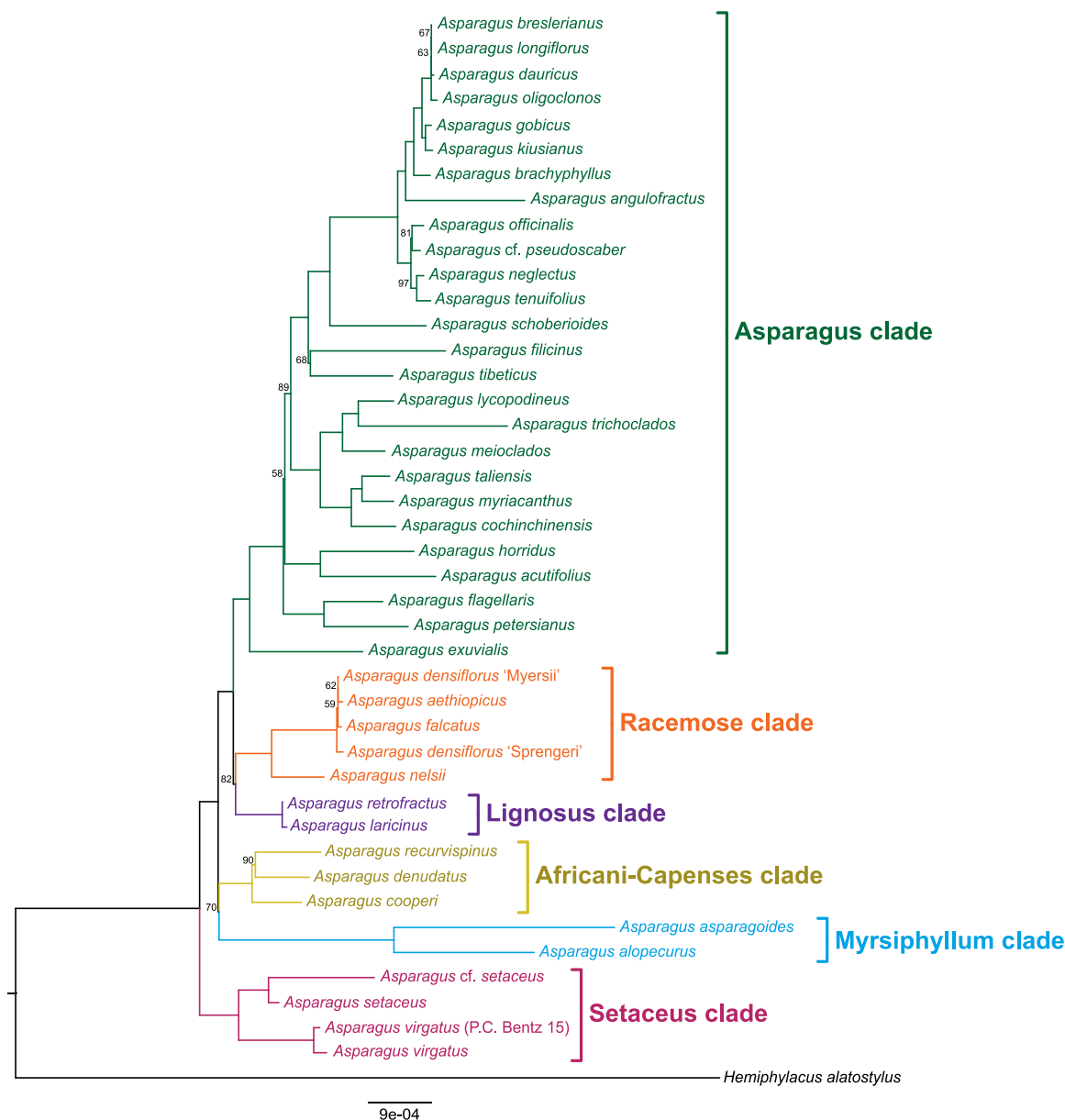


FIGURE 3 Maximum likelihood phylogram based on 77 plastid genes for taxa from all six major clades of *Asparagus*, as hypothesized by Norup et al. (2015). Standard nonparametric bootstrap branch support for all six clades was 100%. Support for relationships within each of these clades were varied, with weaker support usually corresponding to shorter branches. One of the *A. virgatus* samples is labeled with its collection number (P.C. Bentz 15) to differentiate between the two samples for this taxon. Branch values are shown only on branches with <99% support.

analyses that evaluated trees that were not fully bifurcating (right panel in Figure 4; Appendix S5, Figures S5 and S6). When forcing bifurcation, PP support decreased for the following clades in each replicate analysis: single clade with all dioecious taxa (PP = 0.64 and 0.63); *A. officinalis* and *A. cf. pseudoscaber* clade (PP = 0.96 in both); *A. filicinus* and *A. tibeticus* clade (PP = 0.67 and 0.68); clade with Racemosus and Lignosus clades (PP = 0.99 and 0.98); clade containing *A. falcatus* and *A. aethiopicus* (PP = 0.77 in both); clade with Myrsiphyllum and Africani-Capenses clades (PP = 0.57 and 0.54); and clade with *A. denudatus* and *A. recurvispinus* (PP = 0.98 and 0.99) (Appendix S5, Figures S7 and S8).

Divergence time estimation

Time estimates for the stem node of both dioecious clades (i.e., the possible first origin of dioecy, assuming bifurcation) were 2.95–3.78 and 2.81–3.72 Ma, using uniform and lognormal priors, respectively (Table 2). Further, the crown node of both dioecious clades, representing the second possible origin of dioecy was dated at 2.90–3.72 and 2.78–3.67 Ma using uniform and lognormal priors, respectively (Table 2). Stem node estimates for the six clades of *Asparagus* (Figure 3) were as follows when employing uniform and lognormal prior, respectively: 4.55–5.92 and

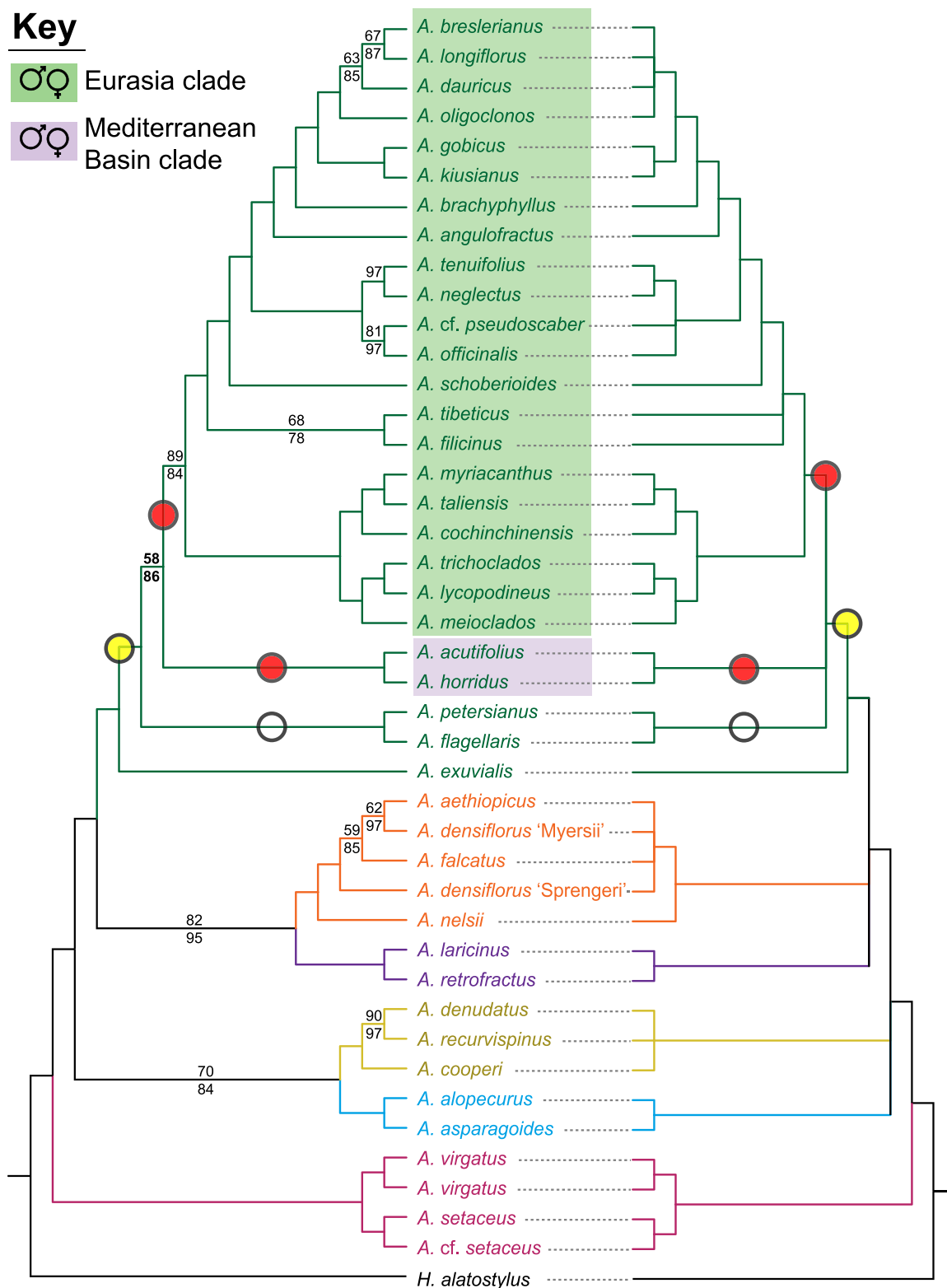


FIGURE 4 Contrasting cladograms for 77 plastid genes from Asparagoideae taxa (*Asparagus* and *Hemiphylacus*) estimated by two analyses: (left) maximum likelihood (ML) with forced bifurcation and (right) Bayesian inference with the probability of polytomies explored. Both trees suggest that dioecy evolved either once (yellow circle) followed by a loss (empty circle), or twice (red circles) in *Asparagus*, based on low support for bifurcations among the Mediterranean Basin dioecious clade (highlighted purple), Eurasia dioecious clade (highlighted green), and a hermaphroditic clade with *A. petersianus* and *A. flagellaris* (left); and strong support for a polytomy among those branches (right). Both scenarios are equally possible in both trees. Colored branches/tips correspond to six major clades of *Asparagus* (Norup et al., 2015) as illustrated in Figure 3 of this study. All nonhighlighted tips are hermaphroditic with monoclinal flowers. Branch values on the ML tree (Left) represent standard nonparametric bootstrap support (top) and ultrafast bootstrap support (bottom) from IQ-TREE (Minh et al., 2020)—shown only on branches with <99% support. All branches in the Bayesian tree (right) had posterior probability support of 1.0 from Phycas (Lewis et al., 2015).

TABLE 2 Divergence time estimates compared across prior treatments in this study and that of previous work for the genus *Asparagus* and Asparagales clades.

Clade	Node	95% HPD age estimates (Ma)				
		Uniform priors ^a	Lognormal priors ^b	Givnish et al. (2018)	McKain et al. (2016)	Chen et al. (2013) ^c
Asparagus clade (Eurasia and Mediterranean Basin) ^d	Crown	2.90–3.72	2.78–3.67	-	-	-
Asparagus clade (Eurasia and Mediterranean Basin) ^e	Stem	2.95–3.78	2.81–3.72	-	-	-
Asparagus clade	Crown	3.46–4.45	3.33–4.39	-	-	-
Asparagus clade	Stem	3.76–4.83	3.62–4.76	-	-	-
<i>Asparagus</i> genus	Stem	9.75–15.04	9.38–14.78	-	12.73–39.58	8.6–25
Asparagoideae	Stem	35.41–44.87	33.36–43.60	39.80–46.24	46.46–80.16	37.4–57.3
Asparagaceae	Stem	46.72–56.68	44.55–55.57	49.43–54.68	65.44–99.09	49.9–67.4
Asparagales	Stem	81.04–92.95	79.56–91.98	111.42–121.02	129.95–172.63	99–113

Note: The *Asparagus* clade corresponds to a clade within the genus *Asparagus* as described by Norup et al. (2015) and shown in Figures 3–5 of this study. HPD = highest density probability interval. Ma = million years ago.

^aResults from BEAST run in this study when uniform prior densities were applied to fossil time calibrations.

^bResults from BEAST run in this study when lognormal prior densities were applied to fossil time calibrations.

^cAge estimates reported for Chen et al. (2013) are median 95% HPD.

^dAge estimates for the crown node of both dioecious clades (i.e., possible dioecy origin number 2), according to the bifurcating BEAST tree.

^eAge estimates for the stem node of both dioecious clades (i.e., possible dioecy origin number 1) according to the bifurcating BEAST tree.

Key

-  Eurasia clade
-  Mediterranean Basin clade
-  Origin(s) of dioecy

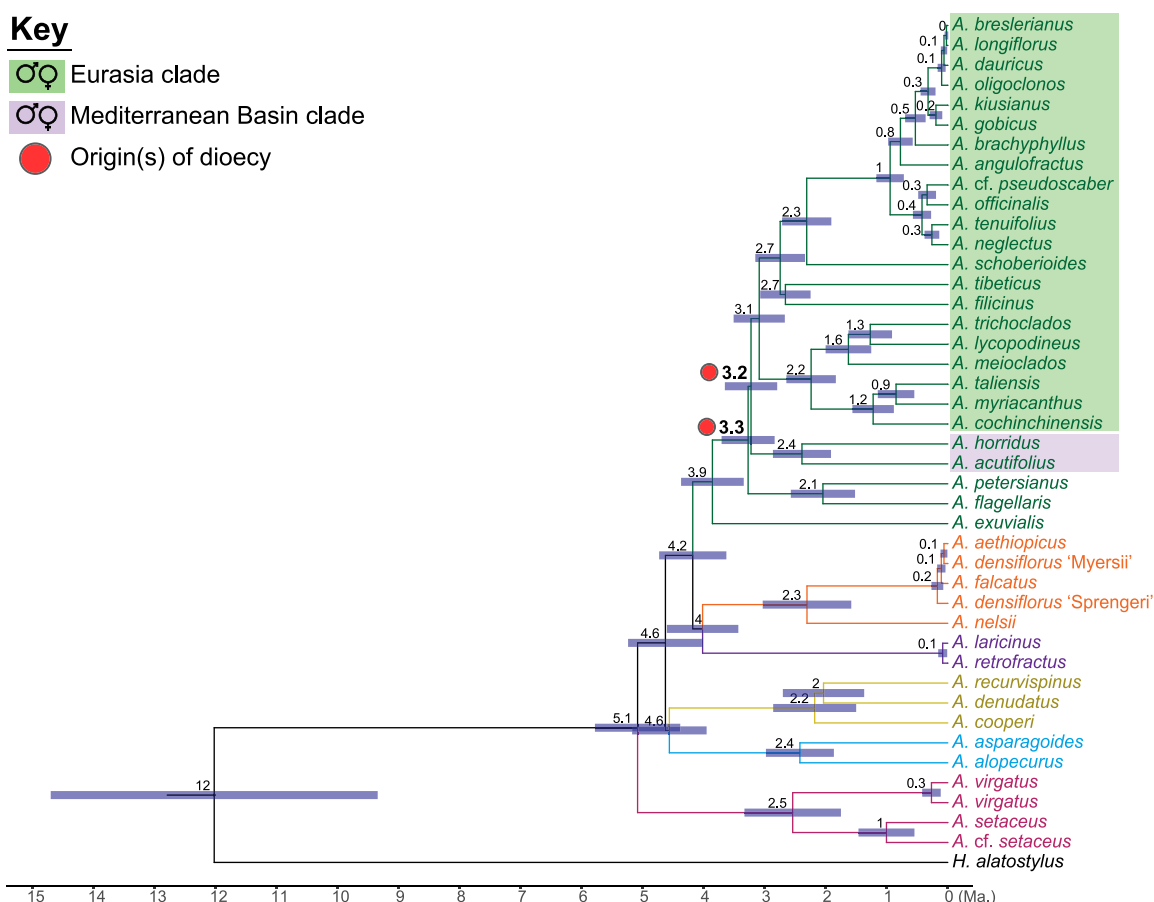


FIGURE 5 Chronogram of Asparagoideae taxa showing relatively young origin(s) of dioecy in the genus *Asparagus*, estimated to have occurred once or twice (due to the short branch length and poor support for a single dioecious clade as shown in Figure 4) approximately 2.78–3.78 million years ago (Ma). Green (Eurasia clade) and purple (Mediterranean Basin clade) highlighted taxa are dioecious and non-highlighted taxa are hermaphroditic with monoclinal flowers. Dark blue node bars represent 95% highest posterior density (HPD) intervals. Branch values are mean age estimates, and scale bar (bottom) is Ma from present. Colored branches/tips correspond to the six major clades of *Asparagus* from Norup et al. (2015).

4.37–5.79 Ma (Setaceus clade), 4.08–5.29 and 3.94–5.20 Ma and 3.94–5.2 Ma (Myrsiphyllum and Africani-Capensis clades), 3.55–4.7 Ma and 3.43–4.62 Ma (Racemose and Lignosus clades), and 3.76–4.83 and 3.62–4.76 Ma (Asparagus clade). The stem node age of the genus *Asparagus* was estimated at 9.75–15.04 and 9.38–14.78 Ma using uniform and lognormal priors, respectively. Age estimates for the stem node of Asparagoideae were 35.41–44.87 (uniform priors) and 33.36–43.60 (lognormal priors) Ma. While the stem node for Asparagaceae was estimated at 46.72–56.68 (uniform priors) and 44.55–55.57 Ma (lognormal priors) and the stem node for Asparagales at 81.04–92.95 (uniform priors) and 79.56–91.98 Ma (lognormal priors). The stem node for all monocots, which used a normal distribution prior in both analyses, was estimated at 132.28–136.18 Ma and 132.15–136.06 Ma in the analyses otherwise featuring uniform and lognormal priors, respectively. For the remaining branches, 95% HPD intervals for divergence time estimates overlapped without differing more than 3 Ma, when comparing results from each prior treatment (Appendix S5, Figures S9 and S10).

DISCUSSION

Geographic distribution of monoclony and dicliny in *Asparagus*

Approximately 78% (111 of 142 spp.) of extant hermaphroditic lineages of *Asparagus* are native to regions south of the Sahara in Africa, while dioecious lineages are only native to the Mediterranean Basin, including northern Africa and Macaronesia, and across Eurasia (Figure 2). The geographic ranges of hermaphroditic and dioecious lineages are largely disjunct, 22% (31) of hermaphroditic species overlap with dioecious relatives in regions bordering the Mediterranean Basin, Macaronesia, the Arabian Peninsula, and India (Figure 2). China represents the center of diversity for dioecious species of *Asparagus*, with 50% (29) of dioecious species occurring there. Whereas the center of diversity for the hermaphroditic species is in southern Africa, with approximately 57% (81) occurring there. Interestingly, about 42% (19) of the hermaphroditic lineages outside of Africa occur in India. Except for *A. albus* and those in India and bordering regions, hermaphroditic species of *Asparagus* do not naturally occur in Eurasia. In contrast, 100% of dioecious lineages occur in Eurasia, including the Mediterranean Basin clade (Figure 4), which also inhabits regions of northern Africa that border the Mediterranean Sea (Figure 2). The geographically widespread *A. racemosus* is the only species reported as native to Australia, although eight other species have naturalized on the continent (Batchelor and Scott, 2006). However, it is possible that the extant, broad distribution of *A. racemosus* is due to historical human introduction given its long-term usage in the Ayurveda and Siddha medicine systems in India and Nepal (Choudhary and Sharma, 2014; Singla and

Jaitak, 2014) and as a popular ornamental and food source (Peters et al., 1992).

Review of sexual systems in *Asparagus*

In reviewing the literature, we found no evidence for gynodioecious, androdioecious, nor monoecious populations of *Asparagus*. We scored 58 (~27% of total species) dioecious and 142 (~66%) hermaphroditic species of *Asparagus* exhibiting diclinous and monoclinal flowers, respectively. We leave open the possibility for these totals to slightly fluctuate because references for 15 species were unattainable or lacked description of sexual system (Appendix S3). Additionally, population-level surveys focused on individual sex phenotypes may reveal inaccuracies in the original species descriptions and monographs used in this study. Some studies incorrectly cite monoecy in *Asparagus* (e.g., Marcellán and Camadro, 1996; Renner and Müller, 2021). Of these two studies, the latter refers to phylogenetic work from Norup et al. (2015) and cites that monoecy has independently evolved several times in *Asparagus*, but zero monoecious species were identified by Norup et al. (2015). The former of the two studies simply refers to *A. densiflorus* as monoecious, which is inaccurate because this species produces hermaphroditic flowers according to the original species description (i.e., Kunth, 1850), subsequent revisions (e.g., Jessop, 1966; Fellingham and Meyer, 1995), and personal observations (P. C. Bentz).

We identified only one peer-reviewed source that makes claims of gynodioecy in *Asparagus* (Norup et al., 2015), which we deemed erroneous for various reasons as described below. Norup et al. (2015) scored species native to Macaronesia as gynodioecious (*Asparagus plocamoides* Webb ex Svent., *Asparagus umbellatus* Link., *Asparagus nesiotis* Svent., and *A. acutifolius*) based on personal observations from the field and herbarium specimens, comments from a third party, and two sources (i.e., Kunth, 1850; Baker, 1875) (Norup et al., 2015). However, none of the samples in question are vouchered (see Table 1 in Norup et al., 2015), so their sexual systems cannot be verified. Also, Baker (1875) wrote that *A. acutifolius* exhibits polygamous flowers (i.e., male, female, or bisexual), and Kunth (1850) cited this species as dioecious, while both Baker (1875) and Kunth (1850) stated that *A. umbellatus* bears hermaphroditic flowers. None of these sources contain information about *A. nesiotis* or *A. plocamoides*. Additionally, all Macaronesian *Asparagus* (except for the dioecious *A. horridus*) were previously diagnosed as hermaphroditic with monoclinal flowers (Valdés, 1979), and *A. acutifolius* is widely accepted as dioecious (e.g., Valdés, 1980; Falavigna et al., 2005; Sica et al., 2005; Kubota et al., 2012; Boubetra et al., 2017; Murase et al., 2017). Further, *A. acutifolius* does not naturally occur in Macaronesia, and the specimen used in Norup et al. (2015) was likely *A. nesiotis* or *A. scoparius* (Boubetra et al., 2017). We scored *A. acutifolius* as dioecious and the other three as hermaphroditic, according to field observations and detailed floral phenotypes described by Valdés (1979).

Based on previous studies and observations as described below, we hypothesize that all sexually dimorphic populations of *Asparagus* can be scored as dioecious or subdioecious (i.e., males, females, and hermaphrodites coexisting). Gynodioecious populations selectively maintain genotypes with distinct female and hermaphrodite phenotypes (Charlesworth, 1989) as opposed to maintenance of genotypes with unstable sex phenotypes (e.g., subdioecy). Dioecy and subdioecy can both superficially appear as gynodioecy when the stable sex phenotype in a population is female and male flowers exhibit remnants of pistil development. In *A. officinalis* flowers, rudimentary organs of the opposite sex commonly develop, and hermaphroditic flower primordia are always present in male flowers (Lazarte and Palser, 1979; Bracale et al., 1990). Further, variation in pistil development exists among and within genotypic male plants of *A. officinalis*, which ranges from nearly no pistil development to fully functional hermaphroditic flowers that produce fruit in andromonoecious males (Galli et al., 1993; Caporali et al., 1994). Rudimentary pistil development is also observed in genotypically male plants of *A. horridus* and *A. acutifolius*, causing their staminate flowers to superficially appear as monoclinal flowers (P. C. Bentz, personal observations). This subdioecious pattern (or “leaky” form of dioecy) is only weakly influenced by the age of dioecy (Käfer et al., 2022), which is relatively young (~2.78–3.78 Ma, see Figure 5 and Table 2) in *Asparagus*. Nonetheless, populations of *Asparagus* formerly described as gynodioecious may be subdioecious with a mix of males, females, hermaphrodites and/or andromonoecious males with varying levels of pistil function—a phenomenon documented in several wild species of *Asparagus* (Tamanyan, 1990) and other sexually dimorphic species (Webb, 1979; Lloyd, 1980; McNeillage, 1991). Field surveys of many plants and multiple populations is necessary to fully understand whether a species’ mating system is hermaphrodite, dioecious, subdioecious, or gynodioecious. Further, pistil fertility trials should be conducted on a large sampling of hermaphroditic-appearing flowers to discern between male and hermaphroditic phenotypes (Arroyo and Raven, 1975; Spigler and Ashman, 2012). It is important to note that although flower morphology (e.g., monoclinal vs. diclinal) can usually be inferred from species descriptions and verified with herbarium specimens, defining sexual systems for a population or species is difficult to infer from herbarium specimens unless multiple individuals of all sex types are vouchered from each population.

Plastome assembly and phylogeny

In this study, we leverage whole plastome assemblies to significantly increase the amount of molecular data used in phylogenetic analyses (i.e., 77 plastid genes) of *Asparagus*, compared to previous studies (e.g., Fukuda et al., 2005; Kubota et al., 2012; Norup et al., 2015). We successfully assembled complete plastomes for all samples except for *A. cf. setaceus* (Burrows & Burrows 7817), due to a lack of

continuous, and/or consistent, read coverage across the entire plastome. Incomplete assembly of the plastome may be a limitation of utilizing off-target reads for plastid sequence recovery (Granados Mendoza et al., 2020). Our phylogenetic analyses agree with others that show strong support for *Hemiphylacus* as sister to *Asparagus* (Norup et al., 2015) and monophyly of the Asparagaceae subfamily Asparagoideae (Fay et al., 2000; Pires et al., 2006; Seberg et al., 2012). We also report significant increases in support for the six major clades of *Asparagus*, as previously proposed to represent the full diversity and geographical distribution of the genus (Norup et al., 2015). Compared to the analyses by Norup et al. (2015) showing overall weak bootstrap support for these clades (i.e., Setaceus clade = 67%; Myrsiphyllum clade = 81%; Africani-Capenses clade = 93%; Lignosus clade = 71%; Racemose clade = 65%; *Asparagus* clade = 89%), all our analyses showed 100% support for all six clades. Notably, we show strong support for the *Asparagus* clade as circumscribed by Norup et al. (2015) (Figures 3 and 4), which includes the hermaphroditic species *A. exuvialis* sister to the rest of the group with stronger bootstrap support (BS) than seen in the previous study (BS = 100% in Figures 3 and 4 vs. BS = 89 in Norup et al., 2015). Additionally, compared to the ubiquitously weak BS support (<66%) for relationships amongst the six major *Asparagus* clades from Norup et al. (2015), we report strong support for the following bifurcations: the Setaceus clade as sister to the remainder of the genus; Myrsiphyllum and Africani-Capenses clades as sister to the Lignosus, Racemose, and *Asparagus* clades; and Lignosus and Racemose clades as sister to the *Asparagus* clade (Figures 3 and 4). Increased taxon sampling within the Myrsiphyllum, Africani-Capenses, Lignosus, and Racemose clades may help resolve relationships among these clades. For now, these unresolved nodes may represent soft polytomies (i.e., those that may be resolved with sufficient data).

Other weakly supported bifurcations in our analyses may represent hard polytomies (i.e., true polytomies supported with sufficient data) resulting from rapid radiations, as indicated by short branches across the backbone of *Asparagus*, a pattern also found in a previous study with much denser taxon sampling (Norup et al., 2015). For example, bifurcations among four species in the Racemose clade (*A. aethiopicus*, *A. densiflorus* ‘Myersii’, *A. densiflorus* ‘Sprengeri’, and *A. falcatus*) were poorly supported in analyses that did not evaluate the likelihood of polytomies, whereas analyses that did evaluate trees with polytomies recovered a well-supported polytomy among these branches (PP = 1) (right panel of Figure 4). Although, it is possible that this well-supported polytomy is an artifact of our limited taxon sampling in this clade and should be tested by phylogenomic analysis with a greater taxon-sampling scheme. Incomplete lineage sorting, in which ancestral variation persists across speciation events, and ancient hybridization may explain the polytomous relationship among these lineages. Incomplete lineage sorting and

hybridization are predicted to occur alongside rapid bursts of diversification (Pease et al., 2016), as is predicted in *Asparagus* (Norup et al., 2015). If hybridization occurred, chloroplast introgression (i.e., chloroplast capture) could cause discordance between the plastid and species tree (Baldwin et al., 2023). However, incomplete lineage sorting, ancient hybridization, and chloroplast capture are not detectable using only the plastome, which is inherited as a single, non-recombining unit (Doyle, 2022). Future investigations should focus on increasing taxon sampling and combining plastome and nuclear sequences in phylogenomic analyses to further explore intra- and interclade relationships across the genus *Asparagus*. Such investigations may also help identify potential source(s) of adaptive variation that may have contributed to rapid species diversification.

Within the *Asparagus* clade, our analyses provide strong support for a polytomy including the Mediterranean Basin dioecious clade, the larger Eurasian dioecious clade, and the hermaphroditic clade with *A. petersianus* and *A. flagellaris* (PP = 1.0; right panel of Figure 4), compared to the poor support from analyses that forced bifurcations (SBS = 58; UFBoot = 86; PP = 65). The nesting of these taxa in the *Asparagus* clade agrees with a previous analysis (Kubota et al., 2012) but disagrees with another (Norup et al., 2015) likely due to misidentification of *A. acutifolius* in the latter (Boubetra et al., 2017). Nonetheless, the polytomous relationship between both dioecious clades leaves open the possibility for independent origins of dioecy in the Mediterranean Basin and Eurasian clades (red circles in Figure 4). Strong support for a monophyletic dioecious clade would support a single origin of dioecy in *Asparagus*. Alternatively, dioecy may have evolved once in an ancestral population of these three lineages emanating from a polytomous node (yellow circle in Figure 4), followed by a loss in the *A. flagellaris* + *A. petersianus* clade (empty circle in Figure 4).

The polytomy estimated at the origin of dioecy in *Asparagus* (right panel in Figure 4) implies that three ancestral lineages failed to coalesce due to a rapid burst of speciation that gave rise to their three ancestral species lineages evolving from a single ancestral species. Whether the speciation process involved a single radiation or two, successive bifurcations cannot be resolved with plastome data alone. These alternative scenarios must be tested through multispecies coalescent analyses including many nuclear loci (e.g., One Thousand Plant Transcriptomes Initiative, 2019). Taxon sampling could also be increased in the Eurasia clade, but no other species are known to associate with the *A. flagellaris* + *A. petersianus* clade, and the only other known species from the Mediterranean Basin clade is *A. aphyllus* (Norup et al., 2015). In a wider context, these two scenarios should be considered when interpreting polytomies in gene trees, especially when gene trees are estimated from large non-recombining loci such as in the plastome.

The nature of polytomies

Polytomies are commonly interpreted as unresolved bifurcations. It is frequently recommended that researchers increase taxon and/or character (e.g., sequence length) sampling to resolve poorly supported branching events due to insufficient data or sampling, low sequence variation, introgression, or ill-suited phylogenetic methods (Whitfield and Lockhart, 2007; Liu et al., 2012). However, these notions discount the possibility of true rapid radiations in which more than two descendant species or haplotypes arise from the same ancestral population or molecule in a species or gene tree, respectively. Evolutionary radiations result in real (hard) polytomies or very short internal branches that are statistically indistinguishable from zero despite sufficient data/power (Maddison, 1989). Polytomies in species trees can be explicitly tested using coalescent-based species tree estimation (Zhang et al., 2018), and polytomies in gene trees (including whole plastome trees) can be assessed using tree inference methods that evaluate the relative support for fully bifurcating trees with trees including polytomies (e.g., Phycas: Lewis et al., 2015). Polytomies in gene trees estimated for single, non-recombining molecules (e.g., plastomes) can arise when more than two lineages that originate from a hub genotype in haplotype network persist and the hub genotype lineage goes extinct. Intraspecific network analyses (e.g., TCS analyses: Templeton et al., 1992; Clement et al., 2000) of plastome sequence data frequently reveal networks with common hub haplotypes linking more than two spoke haplotypes (e.g., Katayama et al., 2012; Lopez et al., 2021). Indeed, TCS network analyses were developed to reconstruct relationships among coexisting ancestral and multiple derived haplotypes (Templeton et al., 1992; Clement et al., 2000). It is certainly plausible that hub haplotypes with more than two spoke haplotype lineages persist over evolutionary time.

Timing for dioecy evolution in *Asparagus*

We estimate that dioecy evolved once or twice (Figure 4) between 2.78 and 3.78 Ma in *Asparagus* (Figure 5). These estimates represent the wider of the two 95% HDP intervals from both divergence time analyses in this study (Table 2). Considering both analyses, if dioecy independently evolved twice, then the second origin would have occurred in the MRCA of the Eurasia clade (Figure 5) approximately 2.78–3.72 Ma (Table 2); while the first would have occurred in the MRCA of the Mediterranean Basin clade (Figure 5) approximately 2.81–3.78 Ma (Table 2). Regardless, these age intervals almost completely overlap, and both scenarios indicate relatively young evolutionary origin(s) of dioecy in *Asparagus*. Since separate sex phenotypes are controlled via the presence of a Y-chromosome in *Asparagus* (Harkess et al., 2017, 2020), age estimates reported in this study support previous hypotheses for the young age of sex chromosomes in the genus and will inform future studies investigating the dynamic evolution of sex chromosomes in this important model system. A previous

review inferred the age of sex chromosomes in *A. officinalis* (Renner and Müller, 2021) from a broad-scale divergence time analysis of four plastid genes and only four *Asparagus* taxa by Chen et al. (2013): *A. densiflorus* (hermaphrodite in Racemose clade: Figure 4), *A. schoberioides*, *A. cochinchinensis*, and *A. oligoclonus* (all three in Eurasian dioecious clade: Figure 4). According to Renner and Müller (2021) the MRCA of these three dioecious taxa diverged from its hermaphroditic ancestor between 1–6 Ma, but it is unclear how these estimates were extrapolated from the original analysis by Chen et al. (2013) because the presented time calibrated tree was missing tip and branch labels (see Figure 3 of Chen et al., 2013). These time estimates were also missing from the main text and online supplemental material from Chen et al. (2013); therefore, we did not include these estimates in Table 2. Either way, inclusion of many more Eurasian samples in our study enables finer-scale comparisons, and our study is the first to test for the age of dioecy in both dioecious clades of *Asparagus*, since Chen et al. (2013) did not include any representatives from the Mediterranean Basin dioecious clade.

Divergence time estimates compared between prior treatments and across similar studies

The two sets of priors (i.e., uniform vs. lognormal probability distributions) applied to fossil time calibrations in BEAST yielded similar divergence time estimates across the phylogeny. Previous studies have presented varying ranges of divergence time estimates for Asparagoideae, Asparagaceae, and Asparagales (Chen et al., 2013; McKain et al., 2016; Givnish et al., 2018). For example, the estimated 95% HPD interval for the crown node age for Asparagoideae was estimated at 12.73–39.58 Ma in one study (McKain et al., 2016), 8.6–25 Ma in another (Chen et al., 2013), and 9.45–14.81 using lognormal priors in the current study (Table 2). However, all other Asparagaceae divergence times estimated by McKain et al. (2016) were older compared to those from the current study and two similar studies (Table 2). Unlike the current and two similar studies (i.e., Chen et al., 2013; Givnish et al., 2018), McKain et al. (2016) did not apply a calibration prior to the stem or crown node of monocots, which may explain the overall older age estimates from analyses, since the age of monocots was constrained in our analyses. All Asparagaceae divergence time estimates reported by Givnish et al. (2018) and Chen et al. (2013) overlap with the 95% HPD intervals presented in this study (Table 2), which is not surprising, since similar time calibration priors were applied across these three studies.

Paleoclimatic oscillations in northern Africa and the origin(s) of dioecy in *Asparagus*

Based on our current understanding of the timing of paleoclimatic oscillations in northern Africa, we speculate that such historical shifts in climate may have influenced the origin

(s) and evolution of dioecy in *Asparagus*. Though the precise timing for the aridification of the Sahara region remains a topic of ongoing research and has been controversial (e.g., see Kroepelin, 2006; Schuster et al., 2006b), it is generally agreed that the region has experienced several cyclical episodes of aridification and humidification and was likely already occurring or began aridifying around the Quaternary ice ages (2–3 Ma) (Ruddiman et al., 1989; Demenocal, 1995; Kroepelin, 2006). Climate model simulations indicate that aridification of northern Africa was largely influenced by shrinkage of the Tethys Sea around 7–11 Ma, leading to periods of arid-humid fluctuations and eventual extreme desert conditions in the Sahara region (Zhang et al., 2014). Recent evidence based on paleosols from the Sahara region suggests that the Sahara Desert formed at least 4.6 Ma (Muhs et al., 2019), and earlier studies infer desert conditions in the region for at least 7 Ma (Schuster et al., 2006a; Senut et al., 2009). All of these studies suggest that arid-humid fluctuations in northern Africa were occurring around the time we estimate dioecy evolved in *Asparagus* (Figure 5: 2.78–3.78 Ma). A biogeographic reconstruction for the genus suggests that hermaphroditic ancestors of extant dioecious lineages of *Asparagus* were involved in multiple independent dispersal events out of southern Africa followed by expansion into Europe and Asia (Norup et al., 2015: Supplementary Figure S5). Considering the geographic distributions of sexual systems in extant *Asparagus* species (Figure 2), it is possible that such climatic oscillations in northern Africa contributed to change in population sizes and shifts in geographical range, potentially leading to selective pressure for transition(s) to dioecy. Under these circumstances, dioecy may evolve partly due to selection for outcrossing to avoid inbreeding depression in fluctuation populations resulting in small effective population sizes and/or founder populations established through long-distance dispersal, and/or colonization of new habitats (Charlesworth, 1999). The maintenance of sexual dimorphism in extant populations may be further explained by increases in fertility via resource allocation between the sexes (e.g., females spending more energy on seed production) (Ross and Weir, 1976). In any case, dioecy clearly evolved in association with range expansion some 3 million years ago and has persisted since then across a broad geographic region.

AUTHOR CONTRIBUTIONS

P.C.B. and J.L.-M. conceived the study and developed experimental designs. P.C.B. conducted experiments, analyzed data, and wrote the manuscript. Z.L., L.Z., J.-B.Y., A.K., and Z.M. contributed to early data analyses. P.C.B., Z.L., L.Z., S.B., J.B., J.-B.Y., A.K., Z.M., and J.L.-M. collected and curated data and contributed to manuscript edits.

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DATA AVAILABILITY STATEMENT

Analysis script and result files are available in Zenodo (<https://zenodo.org/doi:10.5281/zenodo.8436833>). Complete plastome assemblies are available on GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) (accessions: OR571484–OR571520). Individual locus assemblies for *Asparagus cf. setaceus* (Burrows & Burrows 7817) are also in GenBank (accessions: OR541585–OR541660). Raw DNA-Seq reads were deposited to NCBI Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) under the BioProject PRJNA1033351 and China National Center for Bioinformation (<https://ngdc.cnbc.ac.cn>) under BioProjects PRJCA011431 and PRJCA020872.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Taxon sampling and sequence source details.

Appendix S2. Gene sampling and partitioning scheme for plastome phylogenetic Analysis.

Appendix S3. Trait matrix of mating systems and geographic distribution for Asparagoideae taxa.

Appendix S4. Gene assembly and alignment comparisons.

Appendix S5. Phylogenetic and divergence time trees.

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