

RESEARCH ARTICLE

Complete chloroplast genomes of *Asparagus aethiopicus* L., *A. densiflorus* (Kunth) Jessop 'Myers', and *A. cochinchinensis* (Lour.) Merr.: Comparative and phylogenetic analysis with congeners

Kwan-Ho Wong^{1,2}, Bobby Lim-Ho Kong^{2,3}, Tin-Yan Siu¹, Hoi-Yan Wu³, Grace Wing-Chiu But², Pang-Chui Shaw^{2,3,4}, David Tai-Wai Lau^{1,3*}

1 Shiu-Ying Hu Herbarium, School of Life Sciences, The Chinese University of Hong Kong, Hong Kong Special Administrative Region, the People's Republic of China, **2** School of Life Sciences, The Chinese University of Hong Kong, Hong Kong Special Administrative Region, the People's Republic of China, **3** Li Dak Sum Yip Yio Chin R & D Centre for Chinese Medicine, The Chinese University of Hong Kong, Hong Kong Special Administrative Region, the People's Republic of China, **4** State Key Laboratory of Research on Bioactivities and Clinical Applications of Medicinal Plants (The Chinese University of Hong Kong) and Institute of Chinese Medicine, The Chinese University of Hong Kong, Hong Kong Special Administrative Region, the People's Republic of China

* lautaiwai@cuhk.edu.hk



OPEN ACCESS

Citation: Wong K-H, Kong BL-H, Siu T-Y, Wu H-Y, But GW-C, Shaw P, et al. (2022) Complete chloroplast genomes of *Asparagus aethiopicus* L., *A. densiflorus* (Kunth) Jessop 'Myers', and *A. cochinchinensis* (Lour.) Merr.: Comparative and phylogenetic analysis with congeners. PLoS ONE 17(4): e0266376. <https://doi.org/10.1371/journal.pone.0266376>

Editor: Branislav T. Šiler, Institute for Biological Research, University of Belgrade, SERBIA

Received: October 22, 2021

Accepted: March 19, 2022

Published: April 25, 2022

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: <https://doi.org/10.1371/journal.pone.0266376>

Copyright: © 2022 Wong et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: The data that support the findings of this study are openly available in

Abstract

Asparagus species are widely used for medicinal, horticultural, and culinary purposes. Complete chloroplast DNA (cpDNA) genomes of three *Asparagus* specimens collected in Hong Kong—*A. aethiopicus*, *A. densiflorus* 'Myers', and *A. cochinchinensis*—were *de novo* assembled using Illumina sequencing. Their sizes ranged from 157,069 to 157,319 bp, with a total guanine–cytosine content of 37.5%. Structurally, a large single copy (84,598–85,350 bp) and a small single copy (18,677–18,685 bp) were separated by a pair of inverted repeats (26,518–26,573 bp). In total, 136 genes were annotated for *A. aethiopicus* and *A. densiflorus* 'Myers'; these included 90 mRNA, 38 tRNA, and 8 rRNA genes. Further, 132 genes, including 87 mRNA, 37 tRNA, and 8 rRNA genes, were annotated for *A. cochinchinensis*. For comparative and phylogenetic analysis, we included NCBI data for four congeners, *A. setaceus*, *A. racemosus*, *A. schoberioides*, and *A. officinalis*. The gene content, order, and genome structure were relatively conserved among the genomes studied. There were similarities in simple sequence repeats in terms of repeat type, sequence complementarity, and cpDNA partition distribution. *A. densiflorus* 'Myers' had distinctive long sequence repeats in terms of their quantity, type, and length-interval frequency. Divergence hotspots, with nucleotide diversity (Π) ≥ 0.015 , were identified in five genomic regions: *accD-psal*, *ccsA*, *trnS-trnG*, *ycf1*, and *ndhC-trnV*. Here, we summarise the historical changes in the generic subdivision of *Asparagus*. Our phylogenetic analysis, which also elucidates the nomenclatural complexity of *A. aethiopicus* and *A. densiflorus* 'Myers', further supports their close phylogenetic relationship. The findings are consistent with prior generic subdivisions, except for the placement of *A. racemosus*, which requires further study. These *de novo* assembled

GenBank (<https://www.ncbi.nlm.nih.gov>) with the accession number MZ337394, MZ337395 & MZ424304.

Funding: The research work was supported by a donation fund from Wu Jieh Yee Charitable Foundation Limited. The fund has no formal grant number. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

cpDNA genomes contribute valuable genomic resources and help to elucidate *Asparagus* taxonomy.

Introduction

Asparagus, a genus with ca. 300 species [1–6], originated in southern Africa, particularly in the Cape of Good Hope. Some members are now distributed throughout tropical Africa, Eurasia, and Australia [4–11], mostly in arid and sub-arid regions [4–6]. *Asparagus* species have evolved their characteristic morphology as an adaptation to drought and arid environments [2, 4]. Their “true leaves” have been reduced to scales or spines, with the stem-derived organs (“cladodes”) performing photosynthesis [2, 7, 8, 12, 13]. Cladode shape is variable, ranging from acicular, filiform, linear to cordate [2–4, 6, 11, 14–17]. Most species store nutrients and water in rhizomes or root tubers [2, 15–17].

Asparagus species are commercially important worldwide [2, 7, 9, 10, 15, 18, 19], and many are widely used, particularly in medicinal, culinary, and horticultural applications. Here, we first summarise the anthropocentric uses and environmental impacts of some *Asparagus* species and then elucidate the complexity on generic subdivisions and nomenclature of the studied *Asparagus* species.

Medicinal application

Many *Asparagus* species have medicinal value [19–29]. The root tubers of *A. cochinchinensis* (Lour.) Merr., ‘Tiandong’ in Traditional Chinese Medicine, are renowned for their therapeutic functions in nourishing yin, moistening dryness, clearing the heat and engendering fluid [30, 31]. *A. officinalis* L. [20–22, 24–27], *A. setaceus* (Kunth) Jessop [20], *A. filicinus* Buch.-Ham. ex D. Don [24, 28], *A. racemosus* Willd. [19, 21, 25, 27, 29], and *A. schoberioides* Kunth [29] have been used as herbal drugs in different regions for various functions. Root tubers of *A. filicinus* are used as adulterants of *Stemona* Radix to cure tracheitis, pneumonia, coughing, and whooping cough [32–36]. In South African, several *Asparagus* species have been used to treat pulmonary tuberculosis, gonorrhoea, and infertility, while some *Asparagus* species have been used as charm to increase fertility, ensure victory, or fight against witchcraft [21].

Culinary application

Asparagus species are an important culinary resource. Although young shoots of *A. officinalis* L., garden asparagus, are widely sold as a vegetable [1–4, 10, 27, 37], its gene pool is relatively limited [38–40]. It is susceptible to multiple biotic and abiotic stresses, including *Fusarium* rot [41, 42], *Puccinia asparagi* rust [43, 44], purple spot caused by *Stemphylium* [45–47], and stem blight caused by *Phomopsis asparagi* [48], negatively affecting its production and economic value. Attempts to cross *A. officinalis* with its wild relatives, to enhance tolerance to drought, disease, salinity, and acidity [49], have revealed that dioecious, but not monoecious, species could hybridize with it [44, 46, 50–54].

Young shoots of *A. acutifolius* L., *A. aphyllus* L., and *A. albus* L. are also eaten as vegetables [55]. The fruits of *A. racemosus* are edible [56].

Horticultural application

Owing to their distinct morphology, *Asparagus* species, including *A. setaceus*, *A. aethiopicus* L., and *A. densiflorus* (Kunth) Jessop ‘Myers’, have been widely used as ornamental plants [1,

3, 57]. *The European Garden Flora* [57], published in 1986, mentions 24 *Asparagus* species, including *A. setaceus*, *A. aethiopicus*, *A. officinalis*, *A. densiflorus*, *A. filicinus*, *A. asparagoides* (L.) Druce, *A. falcatus* L., and *A. racemosus*. *The New Royal Horticultural Society Dictionary of Gardening* [3], published in 1992, reports the same number of species.

The xeromorphic adaptations of *Asparagus* species are beneficial to the establishment of “Xeroscaping” [58–60], a kind of landscaping that minimises the need for irrigation. The *Pictorial Guide to Plant Resources for Skyrise Greenery in Hong Kong* (Developmental Bureau of the Hong Kong Special Administrative Region Government) [61–63] recommends three *Asparagus* species—*A. cochinchinensis*, *A. aethiopicus* (recorded as *A. densiflorus* ‘Sprengeri’), and *A. densiflorus* ‘Myers’—as skyrise greenery.

Environmental impacts

Global cultivation of *Asparagus* species has promoted the invasiveness of the species, particularly of the horticultural species. The berries of *Asparagus* species are a food source for birds, further promoting their seed dispersal [64]. The invasiveness of *Asparagus* species has been widely recorded in, for instance, Australia [10, 65–67] and the USA [60, 64].

Genus-level taxonomical complexity

Linnaeus first described the genus *Asparagus* in 1753 [68]. Since the publication of the genus *Mysiphyllum* by Willdenow in 1808 [14], generic circumscription of the genus *Asparagus* have been disputed [67, 69, 70]. Based on morphological characters, taxonomists have divided the genus *Asparagus sensu lato* into three genera: genus *Protasparagus* [16, 17, 72] (also known as *Asparagopsis*, an illegitimate homonym [71, 73]); genus *Asparagus sensu stricto* [16, 17, 71–73]; and genus *Myrsiphyllum* [16, 17, 71–73]. The genus *Asparagus sensu lato* has also been divided into three subgenera (subgenus *Asparagopsis*, *Euasparagus*, and *Myrsiphyllum*) [7], or even multiple sections or races [7–9, 15] (S1 Fig). The key morphological characteristics for generic subdivision include the sexual strategy (monoecy or dioecy), perianth segments (free or connate), filaments (free or connate into column), number of ovules per locule (2 or more), cladode shape and arrangement, and presence or absence of spines.

Later evidences and analysis revealed that these subdivisions were not clear-cut. While Malcomber and Demissew [69] advocated to combine these subdivisions into two subgenera under the genus *Asparagus* (subgenus *Asparagus* and subgenus *Myrsiphyllum*), Fellingham and Meyer [70] suggested eliminating the generic subdivisions. It has been stated that “until the phylogenetic relationships within *Asparagus* are investigated in more details, the recognition of any infrageneric groups is problematic” [4].

Norup *et al.* [6] utilised chloroplast and nuclear genome barcode regions (*trnH-psbA*, *trnD-trnT*, 3' *ndhF*, and *PHYC*) in their classification: using 211 accessions representing 119 species, they divided the genus *Asparagus* into six major clades and multiple subclades (S1 Fig).

Species and infraspecific taxonomical complexity

Only one *Asparagus* species, *A. cochinchinensis*, has been recorded as native to Hong Kong. Exotic species that are common in Hong Kong include Sprenger’s asparagus (*A. aethiopicus*), foxtail asparagus (*A. densiflorus* ‘Myers’), lace fern (*A. setaceus*), and garden asparagus (*A. officinalis*). The nomenclature of Sprenger’s asparagus and foxtail asparagus is controversial.

Sprenger's asparagus

The nomenclature of this species is unclear. In 1890, Regel published the name *Asparagus sprengeri* based on cultivated plants growing in Natal, Africa [74, 75]. The epithet *sprengeri* is after Mr. Sprenger, the co-owner of Dammann & Co., which produced this cultivated plant. The name *A. sprengeri* Regel was adopted by Baker (1875) [7] and Geiner (1919) [9]. In 1966, Jessop [15] synonymised *A. sprengeri* Regel under the new combination *A. densiflorus* (Kunth) Jessop, based on morphology and geographical distribution. Since then, it has been commonly recorded as *A. densiflorus*, based on Jessop [1, 3, 5, 10, 11, 57, 64, 70]. It has even been considered a cultivar ('Sprengeri') [1, 57, 64] or a group (the "Sprengeri group") [3] of *A. densiflorus*.

The name *A. aethiopicus* dates from 1767 (S1 Table), when Linnaeus published it in *Species Plantarum* [68]. Eighty-three years later, Kunth [71] transferred the species to the genus *Asparagopsis*. It was later subdivided under the genus *Asparagus* by Baker (in 1875 and 1896) [7, 8] and Jessop (in 1966) [15]. In 1983, Obermeyer [16] transferred it to a new genus *Protasparagus*, because *Asparagopsis* is an illegitimate homonym. Malcomber and Demissew [69] combined the genera *Protasparagus* and *Asparagus* into genus *Asparagus* subgenus *Asparagus* in 1992. Fellingham and Meyer [70], however, cancelled all generic subdivisions three years later, moving it back to the genus *Asparagus*.

Asparagopsis aethiopica (and later *Asparagus aethiopicus*) and *Asparagopsis densiflora* were adopted in parallel for 116 years, from 1850 to 1965. In 1996, Jessop [15] classified both species in the genus *Asparagus* (S1 Table). However, these species are considered highly variable [4, 15]. According to Green (1989) [76], Jessop (1966) [15], Judd (2001) [4], and Straley and Utech (2004) [77], the growth habit of *A. aethiopicus* is more variable, ranging from arching herbs of ca. 1 m in length to scrambling climbers of ca. 7 m in length. In 1986, Green [76] disagreed with Jessop's treatment [15] of *A. sprengeri* as *A. densiflorus*, which is a small-sized species. Green ascribed Jessop's treatment to the omission of *A. densiflorus* from Regel's protologue in *Gartenflora* [75] and to misidentification of cultivated materials, which rarely reach their full potential size as potted plants. Following Judd in 2001 [4], Straley and Utech, in *Flora of North America North of Mexico* (2004) [77], also adopted *A. aethiopicus* for Sprenger's asparagus, stating "*Asparagus densiflorus* (Kunth) Jessop has been misapplied to this species". They considered Sprenger's asparagus to be a cultivar, suggesting the combination as *A. aethiopicus* 'Sprengeri'. On the contrary, Conran, in *Horticultural Flora of South-eastern Australia* [78], treated it as "Sprengeri Group" of *A. aethiopicus*.

The voucher specimens of our research materials were authenticated based on the latest *Asparagus* monograph, *The Genus Asparagus in South Africa* [15], and the *Flora of Hong Kong* [79]. The voucher specimen of Sprenger's asparagus (K. H. Wong 109), collected in Hong Kong, fit the circumscription of *A. aethiopicus* L. in the monograph, based on their habitats, growth habit, and reproductive characteristics. Therefore, we have adopted *A. aethiopicus* L. for Sprenger's asparagus in this study.

Foxtail asparagus

This cultivated plant was named for its foxtail-like branches, which are in narrow cones, assembled by orderly branchlets, densely surrounding the main stem, and gradually elongating from the stem apex [1, 3, 57, 64, 80]. Because of its popularity as an ornamental plant of good performance, the cultivar was named *A. densiflorus* 'Myersii' in the Royal Horticultural Society's *Award of Garden Merit* list [81].

The first binomial name of foxtail asparagus, *Asparagus myersii*, was raised anonymously at an unknown time, while *Asparagopsis densiflora* was validly published in 1850 by Kunth (S1 Table) [71]. The species epithet was named after Mr. Meyers, a nurseryman from East London,

for the introduction of this plant [82]. In 1966, Jessop [15] mentioned that *Asparagus myersii* Hort. “had never been validly published”, treating it as *nomen nudum*. At that time, he combined Kunth’s *Asparagopsis* into *Asparagus* L., deeming this cultivated plant to be a form of *A. densiflorus*. In 1976, this plant was recorded as *A. densiflorus* ‘Myers’ by L. H. Bailey Hortorium in *Hortus III*, [1], treating it as a cultivar of *A. densiflorus*. Since then, this taxonomic treatment has been widely accepted by many taxonomists, horticulturalists, and scientists [3–5, 11, 27, 57, 64, 83].

The spelling of this cultivar epithet occurs in several forms, including the Latin form ‘Myersii’ [57, 80, 81] derived from the species epithet of its *nomen nudum*, the non-Latin form ‘Myers’ [1, 4, 5, 11, 51, 64, 76, 83] and ‘Meyers’ [82, 84, 85]. According to Article 21.6 of the *International Code of Nomenclature of Cultivated Plants* (ICNCP), “the epithet of any name in Latin form published before 1 January 1959, even if it is not validly published under the *International Code of Nomenclature for Algae, Fungi and Plants* (ICN), that meets the requirements for establishment as a cultivar name under this Code (Art. 27.1), may be used as the cultivar epithet, if the plants to which it was applied are now considered to represent a cultivar” [86]. Because these spellings exhibited no ambiguous indication to the same *Asparagus* cultivar as foxtail asparagus, we follow the treatment of some taxonomists and scientists [1, 4, 5, 11, 51, 64, 76, 83], adopting *A. densiflorus* (Kunth) Jessop ‘Myers’ for foxtail asparagus throughout this study.

Provocative molecular evidence: The complete chloroplast genome

Past technical limitations restricted the molecular evidence for classification to short genomic fragments. Technological advancements have made the acquisition of complete genomes, and especially chloroplast genomes, more practicable, affordable, and popular. The chloroplast genome, described as a super-barcode [87–89], is important in studying phylogeny and resolving taxonomical problems [89–92].

Prior to the availability of complete chloroplast DNA (cpDNA) genomes, construction of physical maps of *Asparagus* cpDNA was attempted via Southern hybridisation of total DNA [93, 94]. Lee *et al.* [93] estimated the length of the *A. officinalis* ‘Mary Washington 500W’ cpDNA genome at ca. 155 kb, with two inverted repeats (IRs) of 23 kb each, separated by a 90 kb large single copy (LSC) and a 19 kb small single copy (SSC). The same group constructed the physical maps of cpDNA for another seven *Asparagus* species, *A. schoberioides*, *A. cochinchinensis*, *A. plumosus*, *A. falcatus*, *A. aethiopicus* (recorded as *A. sprengeri*), *A. virgatus*, and *A. asparagoides* [94]. Their results suggest close relationships between these eight species. Despite the high similarity among these species, the cpDNA of *A. falcatus*, *A. sprengeri*, and *A. asparagoides* showed gain of the HindIII restriction site and loss of the XhoI restriction sites. Nucleotide deletion in *rbcL* was detected in *A. cochinchinensis* cpDNA [94].

The first *Asparagus* cpDNA genome (NC_034777.1 = KY364194.1) was reported by Sheng *et al.* in 2017 [95], who assembled and annotated the cpDNA genome of *A. officinalis* ‘Atlas’ (length 156,699 bp); this revealed a quadripartite structure, including a pair of IRs (26,531 bp each), separated by an 84,999 bp LSC and 18,638 bp SSC, very similar to those reported by Lee *et al.* [93]. In 2019, Li *et al.* [96] reported the cpDNA genome of *A. setaceus* (NC_047458.1 = MK950153.1) of 156,978 bp, also quadripartite, and with a pair of IRs (26,513 bp each) separated by 85,311 bp LSC and 18,641 bp SSC. The cpDNA genome of *A. setaceus* is similar to that of *A. officinalis* ‘Atlas’ in terms of structure, gene order, and GC content.

GenBank (National Center for Biotechnology Information; NCBI) currently contains the cpDNA genomes of eight *Asparagus* species: *A. officinalis* (NC_034777.1 = KY364194.1, MT712156.1, LN896355.1, LN896356.1, MT712153.1, MT712155.1, and MT712154.1), *A. setaceus* (NC_047458.1 = MK950153.1 and MT712152.1), *A. cochinchinensis* (MW970105.1 and

MW447164.1), *A. densiflorus* (MT740250.1), *A. dauricus* (MT712151.1), *A. schoberioides* (NC_035969.1 = KX790361.1), *A. racemosus* (NC_047472.1 = MN736960.1), and *A. filicinus* (NC_046783.1 = MK920078.1). This constitutes a small fraction of the genus, leaving a large knowledge gap in the molecular study of *Asparagus*.

We therefore aimed to revisit the phylogenetic relationships between two nomenclaturally confusing species *A. aethiopicus* and *A. densiflorus* 'Myers', using complete cpDNA genomes. This information will be useful in crossbreeding programmes, environmental remediation, and authentication of medicinal materials. Using Illumina sequencing, we *de novo*-assembled the complete chloroplast genomes of *A. aethiopicus*, *A. densiflorus* 'Myers', and *A. cochinchinensis*. We performed comparative and phylogenetic analysis, including congeners, using four cpDNA genomes from GenBank: *A. officinalis* (NC_034777), *A. racemosus* (NC_047472), *A. schoberioides* (NC_035969), and *A. setaceus* (NC_047458). The intra-generic relationships among these seven species were examined and compared to previous generic subdivision. Our analysis helps to elucidate and resolve the taxonomic positions and nomenclature of *A. aethiopicus*, *A. densiflorus* 'Myers', and other congeners.

Materials and methods

Ethics statement

This study was conducted in accordance with Hong Kong Special Administrative Region legislation. Sample collection did not negatively affect the environment in any way.

Plant material and DNA extraction

Individuals of the studied species were collected from the Chinese University of Hong Kong (Table 1 and Fig 1). Fresh and healthy cladodes were stored at -80°C in a freezer immediately after collection. Voucher specimens were deposited at the Shiu-Ying Hu Herbarium (herbarium code: CUHK).

Total genomic DNA was extracted from 0.2 g of frozen cladode using the DNeasy Plant Pro Kit (Qiagen Co., Hilden, Germany) according to the manufacturer's instructions. Prior to the sequencing conducted by Novogene Bioinformatic Technology Co. Ltd. (<http://en.novogene.com/>, Beijing, China), DNA quantity and quality were assessed using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, MA, USA) and 1% agarose gel electrophoresis, respectively.

cpDNA genome sequencing, assembly, and annotation

A paired-end library with an insert-size of 150 bp was constructed and sequenced on a Nova-Seq 6000 platform (Illumina Inc. San Diego, CA, USA). Raw reads were quality-trimmed using CLC Assembly Cell 5.1.1 (CLC Inc., Denmark), with Phred < 33 . The trimmed reads were assembled into contigs using the CLC *de novo* assembler. Gaps were filled using Gapcloser in SOAPdenovo 3.23 to form contigs, then retrieved and ordered using NUCmer 3.0 [97]. The ordered contigs were aligned against reference chloroplast genomes. Based on

Table 1. Information about the *Asparagus* specimens deposited at the Shiu-Ying Hu Herbarium.

Species	Collector no.	Inventory no.	Sheet no.	GPS location
<i>Asparagus aethiopicus</i> L.	K. H. Wong 109	CUSLSH2801	CUHK05891	22.420786, 114.208312
<i>Asparagus densiflorus</i> (Kunth) Jessop 'Myers'	K. H. Wong 092	CUSLSH2773	CUHK05890	22.419994, 114.207354
<i>Asparagus cochinchinensis</i> (Lour.) Merr.	K. H. Wong 107	CUSLSH2799	CUHK05892	22.421524, 114.207135

<https://doi.org/10.1371/journal.pone.0266376.t001>



Fig 1. Photos of three *Asparagus* plants collected at the Chinese University of Hong Kong. A,B: *A. aethiopicus*. A. Plant climbing under *Ficus microcarpa* L. f. and twining with *Passiflora suberosa* L. B. Flowers and cladodes. C,D: *A. cochinchinensis*. C. Plant straggling on ground. D. Cladodes. E,F,G: *A. densiflorus* 'Myers'. E. Plant growing in a concrete pot. F. Flowers and cladodes. G. Fruits and branch apices.

<https://doi.org/10.1371/journal.pone.0266376.g001>

phylogenetic proximity, *A. setaceus* (NC_047458) was selected as the reference genome for *A. aethiopicus* and *A. densiflorus* 'Myers', whereas *A. schoberioides* (NC_035969) was used for *A. cochinchinensis*. The aligned contigs were assembled into a complete cpDNA genome for each species.

Gene annotation of cpDNA was performed on the GeSeq platform (<https://chlorobox.mpimp-golm.mpg.de/geseq.html>) [98] based on the GenBank chloroplast genomes. *A. aethiopicus* and *A. densiflorus* 'Myers' were annotated in reference to *A. setaceus* (NC_047458) and *A. racemosus* (NC_047472), while *A. cochinchinensis* was annotated in reference to *A. schoberioides* Kunth (NC_035969) and *A. officinalis* L. (NC_034777). Manual adjustments, including editing the start and stop positions of genes and introns, were made where necessary. The circular genomic map was visualised by OrganellarGenomeDRAW (OGDRAW, <https://chlorobox.mpimp-golm.mpg.de/OGDraw.html>) [99]. The assembled and annotated chloroplast genomes of *A. aethiopicus*, *A. densiflorus* 'Myers', and *A. cochinchinensis* were submitted to GenBank (accession numbers MZ337394, MZ337395, and MZ424304, respectively).

Repeat-sequence analysis

To compare the three newly assembled cpDNA genomes with chloroplast genomes of other *Asparagus* species, four cpDNA genomes (NC_034777, NC_047472, NC_035969, and NC_047458) were downloaded from GenBank. Repeat motifs, including simple sequence repeats (SSRs) and long sequence repeats (LSRs), were sequentially identified using the Micro-Satellite identification tool (MISA, <https://webblast.ipk-gatersleben.de/misa/index.php?action=1>) [100] and REPuter (<https://bibiserv.cebitec.uni-bielefeld.de/reputer>) [101]. We

screened for SSRs with at least 10, 5, 4, 3, 3, and 3 repeats, respectively, for mono-, di-, tri-, tetra-, penta-, and hexa-nucleotides. LSRs, including forward, reverse, complement, and palindromic sequences, were detected with a maximum computed repeat size of 50 bp and minimal repeat size of 30 bp.

Comparative genome analysis

For structural comparison of the seven cpDNA genomes, we used mVISTA software (<https://genome.lbl.gov/vista/mvista/submit.shtml>) [102] to visualise the full alignment with annotation, using the *A. aethiopicus* cpDNA genome as the reference. The shuffle-LAGAN alignment programme [103] was used.

To compare the size and type of IR border genes, IRScope (<https://irscope.shinyapps.io/irapp/>) [104] was used to visualise the junction sites of the seven cpDNA genomes. Junction gene positions and sizes were verified, and the diagram was redrawn manually.

To investigate divergence hotspots, the seven studied cpDNA genomes were first aligned using MAFFT 7 (<https://mafft.cbrc.jp/alignment/server/>) [105]. Sliding window analysis was conducted using DNA Sequence Polymorphism (DnaSP) 6.12.03 [106], which calculates the nucleotide diversity value (Pi) of the aligned cpDNA. The window length and step size were set to 600 and 200 bp, respectively.

Phylogenetic analysis

The complete cpDNA genomes of the seven *Asparagus* species, with one outgroup species, *Hyacinthoides non-scripta* (L.) Chouard ex Rothm. (NC_046498), were used to construct maximum likelihood (ML) phylogenetic trees using the MEGA-X software [107], with 1000 bootstrap replicates for each tree. The best-fit model of nucleotide substitution, with the lowest Bayesian Information Criterion (BIC) scores, was calculated via ML model selection in MEGA-X. Respective trees were constructed from the aligned sequences of (i) complete cpDNA genome, (ii) protein coding (CDS) regions (excluding introns), (iii) LSC, (iv) SSC, and (v) IRs.

Results

Asparagus cpDNA genomes features

Illumina NovaSeq 6000 sequencing generated 3.2 Gb, 3.1 Gb, and 2.8 Gb raw data for *A. aethiopicus*, *A. densiflorus* 'Myers', and *A. cochinchinensis*, respectively. The cpDNA genomes were assembled with a coverage of 173x for *A. aethiopicus*, 164x for *A. densiflorus* 'Myers', and 381x for *A. cochinchinensis*.

The three newly assembled cpDNA genomes were relatively conserved in terms of length, gene order, gene content, and structure. The cpDNA genome of *A. densiflorus* 'Myers' was the largest (157,139 bp), followed by *A. aethiopicus* (157,069 bp), and *A. cochinchinensis* (156,319 bp; Table 2 and Fig 2). The cpDNA genomes exhibited the quadripartite structure typical of angiosperms. Their LSCs ranged from 84,598 to 85,350 bp in length and their IRs from 26,518 to 26,573 bp. The SSC was 18,677 bp for both *A. aethiopicus* and *A. densiflorus* 'Myers', and 18,685 bp for *A. cochinchinensis*.

Identical numbers and types of genes were annotated in *A. aethiopicus* and *A. densiflorus* 'Myers'. One hundred and thirty-six genes were successfully annotated, including 90 protein-coding (mRNA) genes, 38 transcription- and translation-related RNA (tRNA) genes, and 8 ribosomal RNA (rRNA) genes. For *A. cochinchinensis*, 132 genes were annotated, including 87

Table 2. Summary on the cpDNA genome structure of the seven *Asparagus* species.

	<i>A. aethiopicus</i>	<i>A. densiflorus</i> 'Myers'	<i>A. cochinchinensis</i>	<i>A. officinalis</i>	<i>A. racemosus</i>	<i>A. schoberioides</i>	<i>A. setaceus</i>
Accession no.	MZ337394	MZ337395	MZ424304	NC_034777	NC_047472	NC_035969	NC_047458
Total length (bp)	157,069	157,139	156,319	156,699	156,742	156,875	156,978
LSC (bp)	85,246	85,350	84,598	84,999	84,989	84,928	85,311
SSC (bp)	18,677	18,677	18,685	18,638	18,619	18,685	18,641
IR (bp)	26,573	26,556	26,518	26,531	26,567	26,631	26,513
Total number of genes	136	136	132	133	130	132	135
mRNA	90	90	87	88	86	88	90
tRNA	38	38	37	37	36	36	37
rRNA	8	8	8	8	8	8	8
Pseudogene (Ψ)	1 ^a	1 ^a	0	7 ^b	1 ^a	1 ^a	1 ^a
1-intron gene	20	21	21	21	21	20	19
2-introns gene	2	2	2	2	2	2	2
Total GC content (%)	37.49	37.49	37.54	37.59	37.55	37.57	37.48
GC content in LSC (%)	35.44	35.43	35.54	35.60	35.53	35.55	35.46
GC content in SSC (%)	31.30	31.31	31.38	31.50	31.43	31.51	31.45
GC content in IR (%)	42.94	42.93	42.90	42.92	42.92	42.93	42.85

^a *ycf1*^b *ycf1*, *ycf15* (x2), *ycf68* (x2), *infA*, *rps19*.<https://doi.org/10.1371/journal.pone.0266376.t002>

mRNA genes, 37 tRNA genes, and 8 rRNA genes. The genes were classified into three categories and 18 functions (Table 3).

The pseudogene *ycf1* occurred in *A. aethiopicus* and *A. densiflorus* 'Myers' but was not detected in *A. cochinchinensis*. *A. densiflorus* 'Myers' and *A. cochinchinensis* had 21 intron-containing genes, whereas *A. aethiopicus* had 20. All three cpDNA genomes had two genes comprising two introns (Table 4). For *A. aethiopicus* and *A. densiflorus* 'Myers', 20 genes were duplicated in IRs. In contrast, only 19 genes were duplicated in the IRs for *A. cochinchinensis*, because *ycf68* was absent from this genome.

The cpDNA genomes of the three species were comparable in terms of GC content (Table 2). In total, 37.5% of the GC bases were detected in all three cpDNA genomes; 35.4–35.5%, 31.3–31.4%, and 42.9% of the GC content was detected in LSCs, SSCs, and IRs, respectively. Among the three cpDNA genomes, *A. cochinchinensis* had the highest GC content (37.54%), with 35.54% in LSCs and 31.38% in SSCs, whereas *A. aethiopicus* had the highest IR GC content (42.94%).

Simple sequence repeat analysis

The SSR number, type, content, and distribution were similar in the seven cpDNA genomes. The number of SSRs ranged from 80 (*A. schoberioides*) to 88 (*A. aethiopicus* and *A. officinalis*) (Fig 3).

Each cpDNA sample contained mono-, di-, tri-, or tetra-nucleotides. Three of the seven cpDNA genomes contained pentanucleotides, whereas the other four contained hexanucleotides. The most common class of SSRs was mononucleotides, ranging from 47 in *A. densiflorus* 'Myers' to 57 in *A. officinalis*. Dinucleotides were the second most common, ranging from 12 in *A. racemosus* to 15 in *A. aethiopicus* and *A. densiflorus* 'Myers'. Tetranucleotides were the third most common, ranging from 10 in *A. schoberioides* to 13 in *A. aethiopicus* and *A. densiflorus* 'Myers'. Trinucleotides repeats were the least common, with five each in *A.*

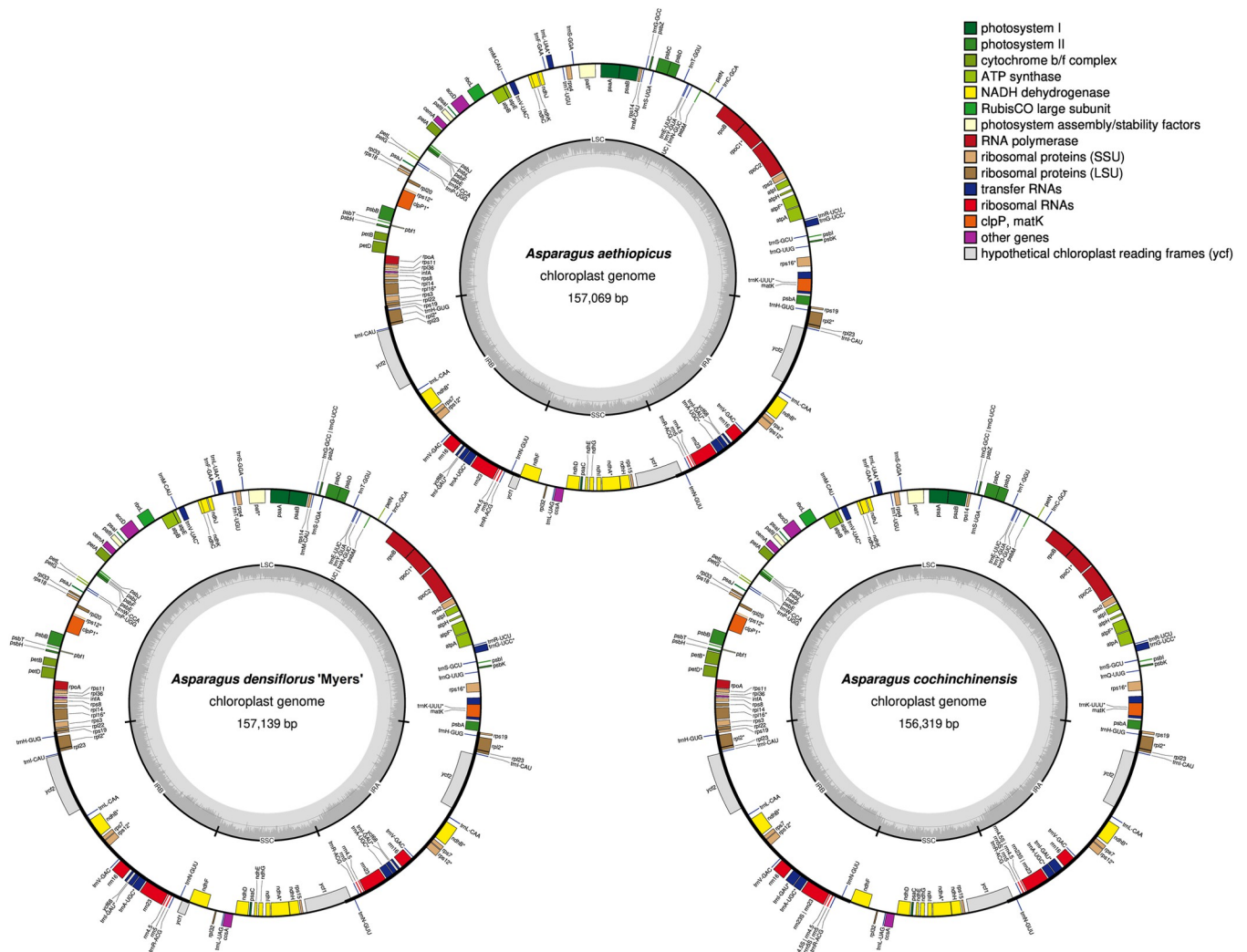


Fig 2. Chloroplast genome map of *A. aethiopicus* L., *A. densiflorus* (Kunth) Jessop 'Myers', and *A. cochinchinensis* (Lour.) Merr. Genes are colour-coded based on their functions shown in the key. Genes located outside of the outer circle are transcribed anticlockwise, while those inside are transcribed clockwise. In the inner circle, the gradient in dark grey represents GC content, whereas light grey represents AT content.

<https://doi.org/10.1371/journal.pone.0266376.g002>

cochinchinensis, *A. officinalis*, *A. racemosus*, and *A. schoberioides*, and seven each in the other species. One or two pentanucleotide or hexanucleotide repeats were found in each of the seven genomes.

Considering sequence complementarity, most of the SSRs were A/T (adenosine/thymine) repeats, ranging from 46 in *A. densiflorus* 'Myers' to 55 in *A. officinalis* (Fig 4). AT/AT repeats were the second most common, from 9 in *A. racemosus* to 12 in *A. aethiopicus* and *A. densiflorus* 'Myers'. AAAT/ATTT repeats were the third most common, at 4 in *A. officinalis*, 6 in *A. schoberioides*, and 7 in the other cpDNA genomes.

For the seven genomes, 87.59% of the SSRs comprised entirely adenosine and thymine, with at most 2 bp of guanine and cytosine in the GC-containing SSRs. The dominance of A/T base pairs and low frequency of G/C base pairs in SSRs are consistent with the observations made by Sheng *et al.* [95].

Table 3. Genes annotated in the complete cpDNA genomes of *A. aethiopicus* L., *A. densiflorus* (Kunth) Jessop ‘Myers’, and *A. cochinchinensis* (Lour.) Merr.

Gene category	Gene functions	Gene names
Photosynthesis-related genes	Rubisco	<i>rbcL</i>
	Photosystem I	<i>psaA, psaB, psaC, psaI, psaJ</i>
	Assembly/ stability of photosystem I	<i>psaI, psfII, pbf1</i>
	Photosystem II	<i>psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbI, psbJ, psbK, psbL, psbM, psbT, psbZ</i>
	ATP synthase	<i>atpA, atpB, atpE, atpF, atpH, atpI</i>
	Cytochrome b/f complex	<i>petA, petB, petD, petG, petL, petN</i>
	Cytochrome c synthesis	<i>ccsA</i>
	NADPH dehydrogenase	<i>ndhA, ndhB[%], ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhJ, ndhK</i>
Transcription- and translation-related genes	Transcription	<i>rpoA, rpoB, rpoC1, rpoC2</i>
	Ribosomal protein	<i>rpl2[%], rpl14, rpl16, rpl20, rpl22, rpl23[%], rpl32, rpl33, rpl36, rps2, rps3, rps4, rps7[%], rps8, rps11, rps12[%], rps14, rps15, rps16, rps18, rps19[%]</i>
	Translation initiation factor	<i>infA</i>
RNA genes	Ribosomal RNA	<i>rrn16[%], rrn23[%], rrn4.5[%], rrn5[%]</i>
	Transfer RNA	<i>trnA-UGC[%], trnC-GCA, trnE-UUC, trnF-GAA, trnG-GCC, trnG-UCC[*], trnH-GUG[%], trnI-CAU[%], trnI-GAU[%], trnK-UUU, trnL-CAA[%], trnL-UAA, trnL-UAG, trnM-CAU[§], trnN-GUU[%], trnN-GUC, trnP-UGG, trnQ-UUG, trnR-ACG[%], trnR-UCU, trnS-GCU, trnS-GGA, trnS-UGA, trnT-GGU, trnT-UGU, trnV-GAC[%], trnV-UAC, trnW-CCA, trnY-GUA</i>
Miscellaneous group	Maturase	<i>matK</i>
	Inner membrane protein	<i>cemA</i>
	ATP-dependent protease	<i>clpP1</i>
	Acetyl-CoA carboxylase	<i>accD</i>
	Unknown functions	<i>ycf1[@], ycf2[%], ycf68[#]</i>

[%] Duplicated in inverted repeat regions

^{*} Duplicated in large single copies of *A. densiflorus* ‘Myers’ and *A. cochinchinensis*; appeared once in *A. aethiopicus*

[§] Duplicated in large single copies of *A. aethiopicus* and *A. densiflorus* ‘Myers’; appeared once in *A. cochinchinensis*

[@] *ycf1* was functional in all three species, but the *ycf1* pseudogene was absent from *A. cochinchinensis*

[#] Duplicated in inverted repeat regions of *A. aethiopicus* and *A. densiflorus* ‘Myers’; absent from *A. cochinchinensis*.

<https://doi.org/10.1371/journal.pone.0266376.t003>

The cpDNA genomes demonstrated similar proportional distributions of SSRs within the quadripartite structure (Fig 5), with most (ca. two-thirds) found in LSC regions and one-fifth and one-tenth, respectively, found in SSC and IR regions.

Long sequence repeat analysis

The species differed significantly in the LSR analysis, particularly for *A. densiflorus* ‘Myers’ (Figs 6 and 7): for the other six genomes, there were 2 LSRs (*A. officinalis* and *A. schoberioides*) to 5 LSRs (*A. cochinchinensis*), whereas *A. densiflorus* ‘Myers’ had 34 LSRs, almost 10-fold the average in the others.

All four types of LSRs (forward, reverse, palindromic, and complement repeat) were detected. Notably, the genomes contained from 1 (*A. officinalis*) to 3 (*A. densiflorus* ‘Myers’) types of LSRs. Palindromic repeats were the most common LSR type: of the 29 palindromic repeats, *A. densiflorus* ‘Myers’ had 17. Forward repeats were second, occurring in five of the species, excluding *A. officinalis* and *A. racemosus*. Of the 22 forward repeats, *A. densiflorus* ‘Myers’ had 16. *A. densiflorus* ‘Myers’ and *A. racemosus* had 1 reverse repeat and 1 complement repeat, respectively.

The minimum repeat size was set to 30 bp. The longest LSR detected by REPuter was 56 bp. LSRs were detected at lengths of 30, 31, 32, 33, 34, 35, 36, 38, 39, 46, 47, 49, 52, 54, and 56 bp.

Table 4. Intron-containing genes in the chloroplast genomes of seven *Asparagus* species.

	<i>A. aethiopicus</i>	<i>A. densiflorus</i> 'Myers'	<i>A. cochinchinensis</i>	<i>A. officinalis</i>	<i>A. racemosus</i>	<i>A. schoberioides</i>	<i>A. setaceus</i>	Location
Accession no.	MZ337394	MZ337395	MZ424304	NC_034777	NC_047472	NC_035969	NC_047458	/
<i>trnK-UUU</i>	0	1	1	1	1	1	0	LSC
<i>rps16</i>	1	1	1	1	1	1	1	LSC
<i>trnG-UCC</i> ^B	1	1	1	1	1	1	ABS	LSC
<i>atpF</i>	1	1	1	1	1	1	1	LSC
<i>rpoC1</i>	1	1	1	1	1	1	1	LSC
<i>ycf3/pafI</i> ^C	2	2	2	2	2	2	2	LSC
<i>trnL-UAA</i>	1	1	1	1	1	ABS	1	LSC
<i>trnV-UAC</i>	1	1	1	1	1	1	1	LSC
<i>clpP</i>	2 ^A	2 ^A	2 ^A	2	2	2	2	LSC
<i>petB</i>	1	1	1	1	1	1	1	LSC
<i>petD</i>	1	1	1	1	1	1	1	LSC
<i>rpl16</i>	1	1	1	1	1	1	1	LSC
<i>rpl2</i> *2	1	1	1	1	1	1	1	IR _A + IR _B
<i>ndhB</i> *2	1	1	1	1	1	1	1	IR _A + IR _B
<i>rps12</i> *2	1	1	1	1	1	1	1	IR _A + IR _B + LSC
<i>trnI-GAU</i> *2	1	1	1	1	1	1	1	IR _A + IR _B
<i>trnA-UGC</i> *2	1	1	1	1	1	1	1	IR _A + IR _B
<i>ndhA</i>	1	1	1	1	1	1	1	SSC

0—No intron; 1—1 intron; 2—2 introns; ABS—Gene absent.

^A Annotated as *clpP1*.

^B Located in the region 9167–9994 bp; for NC 047458, *trnG-UCC*, at 36924–36994 bp, had no intron.

^C *pafI* was annotated in *A. aethiopicus*, *A. densiflorus* 'Myers', and *A. cochinchinensis*.

<https://doi.org/10.1371/journal.pone.0266376.t004>

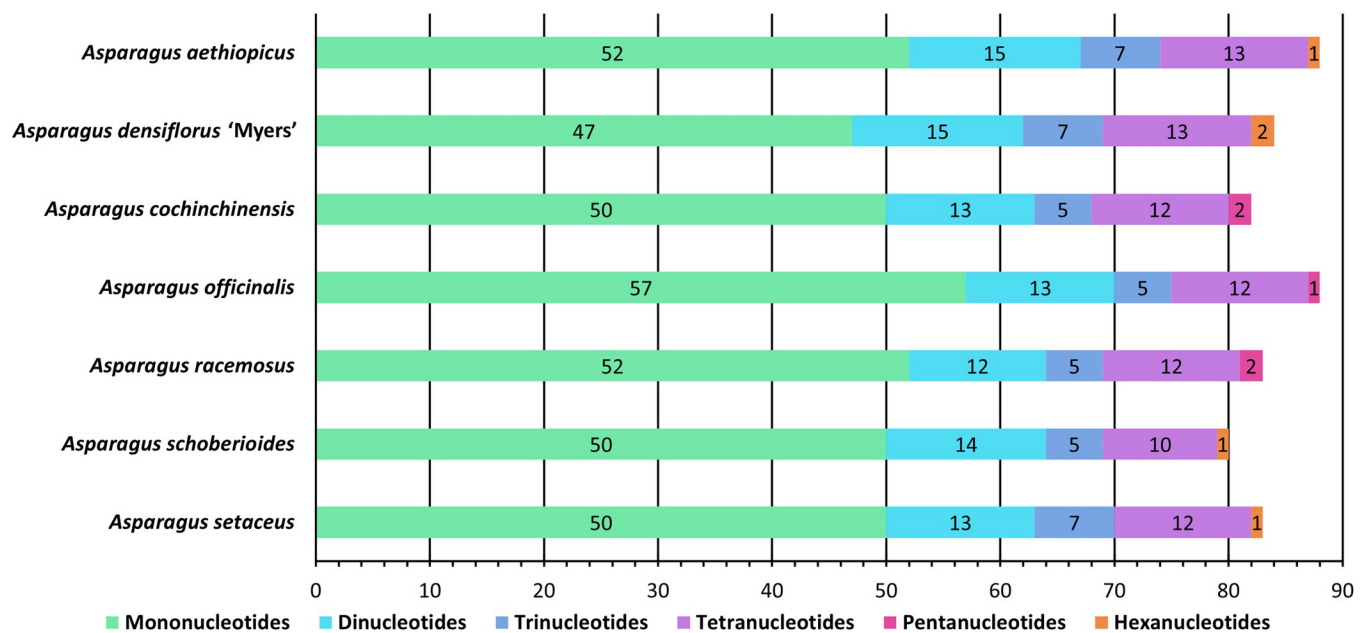


Fig 3. Simple sequence repeat class distribution.

<https://doi.org/10.1371/journal.pone.0266376.g003>

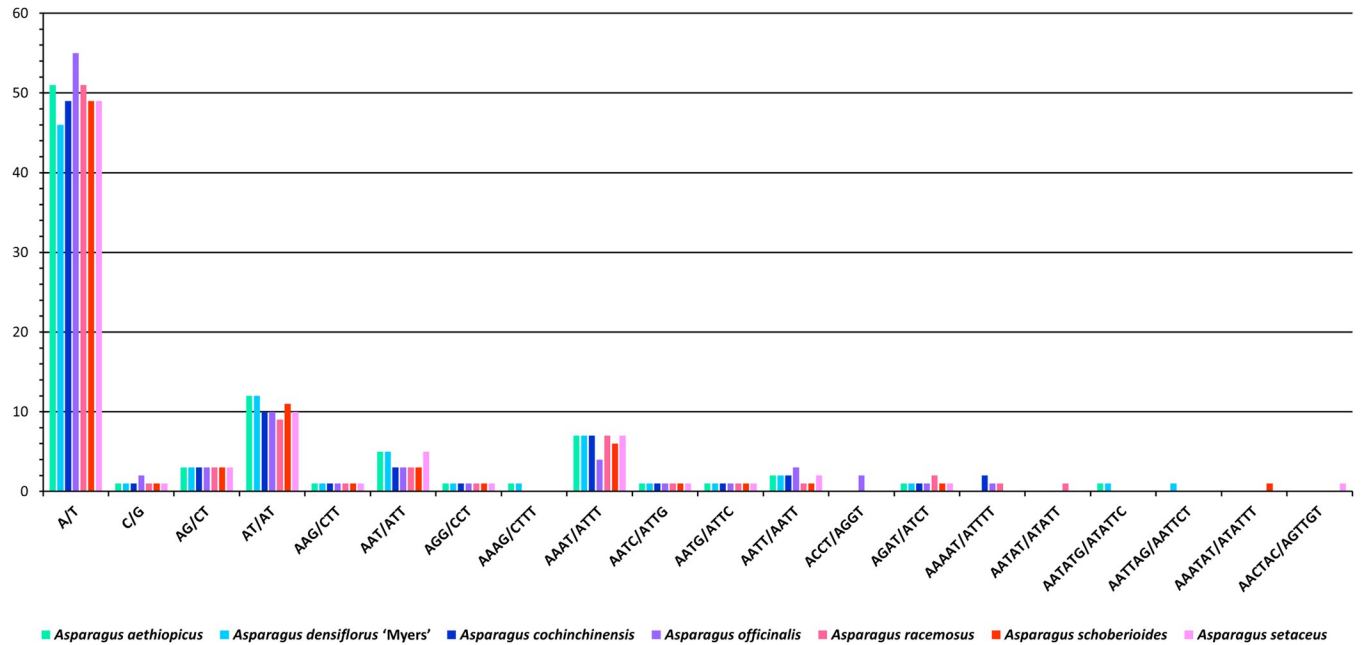


Fig 4. Simple sequence repeat frequency related to sequence complementarity.

<https://doi.org/10.1371/journal.pone.0266376.g004>

Fig 7 represents their frequencies in three intervals: (i) 30–39 bp, (ii) 40–49 bp, and (iii) 50–56 bp. LSRs of 30–39 bp and 50–56 bp occurred in all three species, whereas only *A. densiflorus* ‘Myers’ has LSRs of 40–49 bp (six, in total). LSRs of 30–39 bp were the most common, with 39

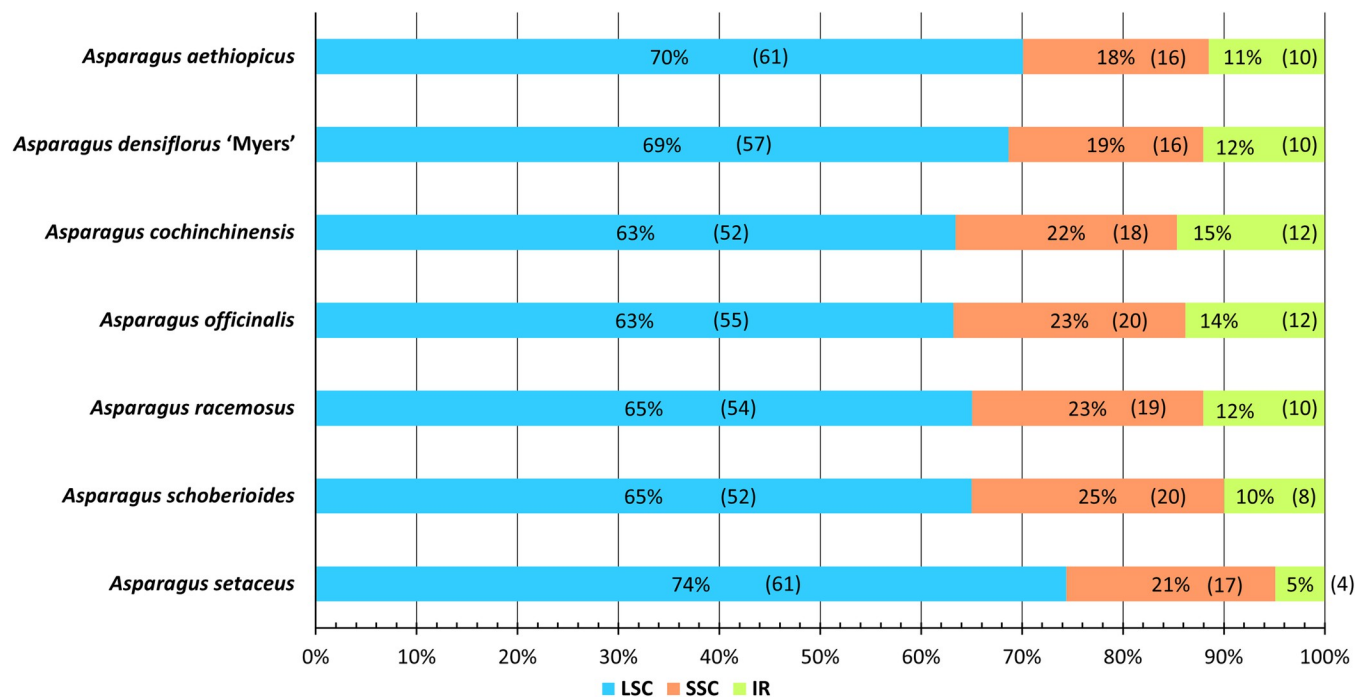


Fig 5. Simple sequence repeat distribution in the quadripartite cpDNA structure. The percentages for each region are shown in the middle of each bar. The numbers in brackets are the actual numbers of SSRs distributed in the indicated cpDNA regions.

<https://doi.org/10.1371/journal.pone.0266376.g005>

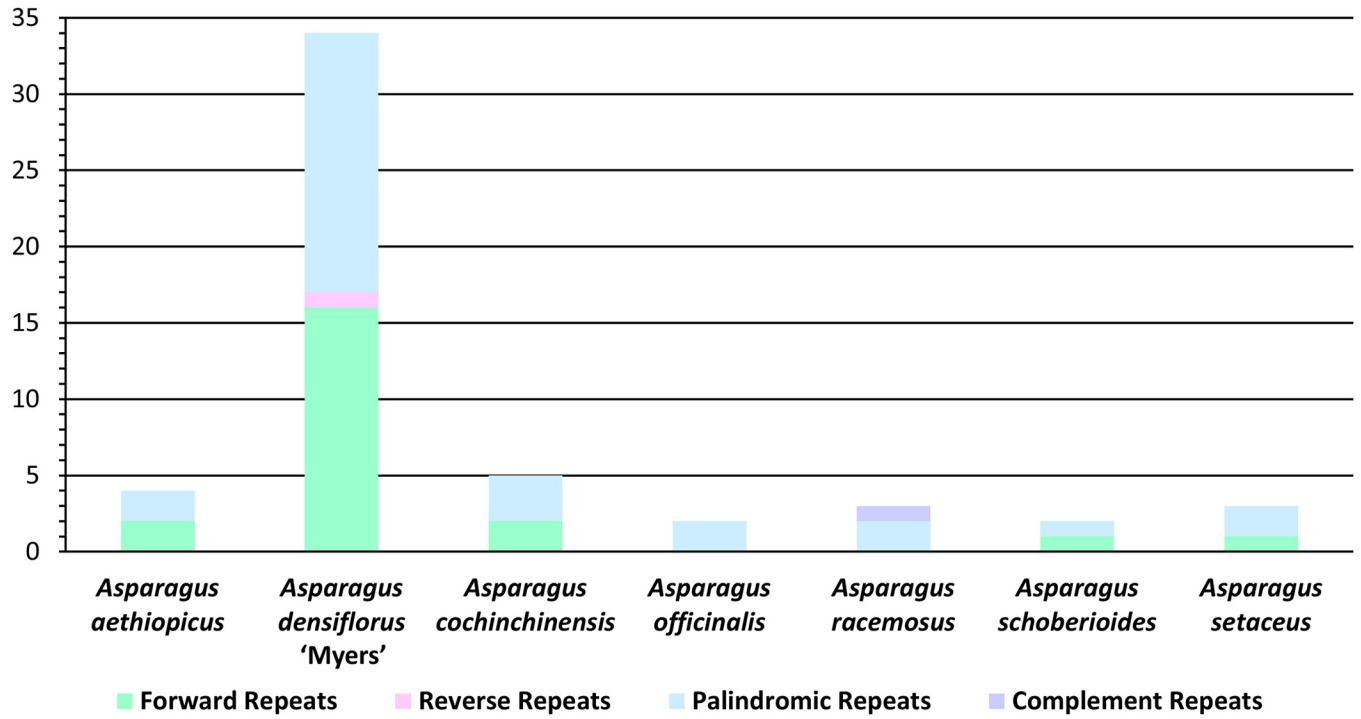


Fig 6. Types of long sequence repeats.

<https://doi.org/10.1371/journal.pone.0266376.g006>

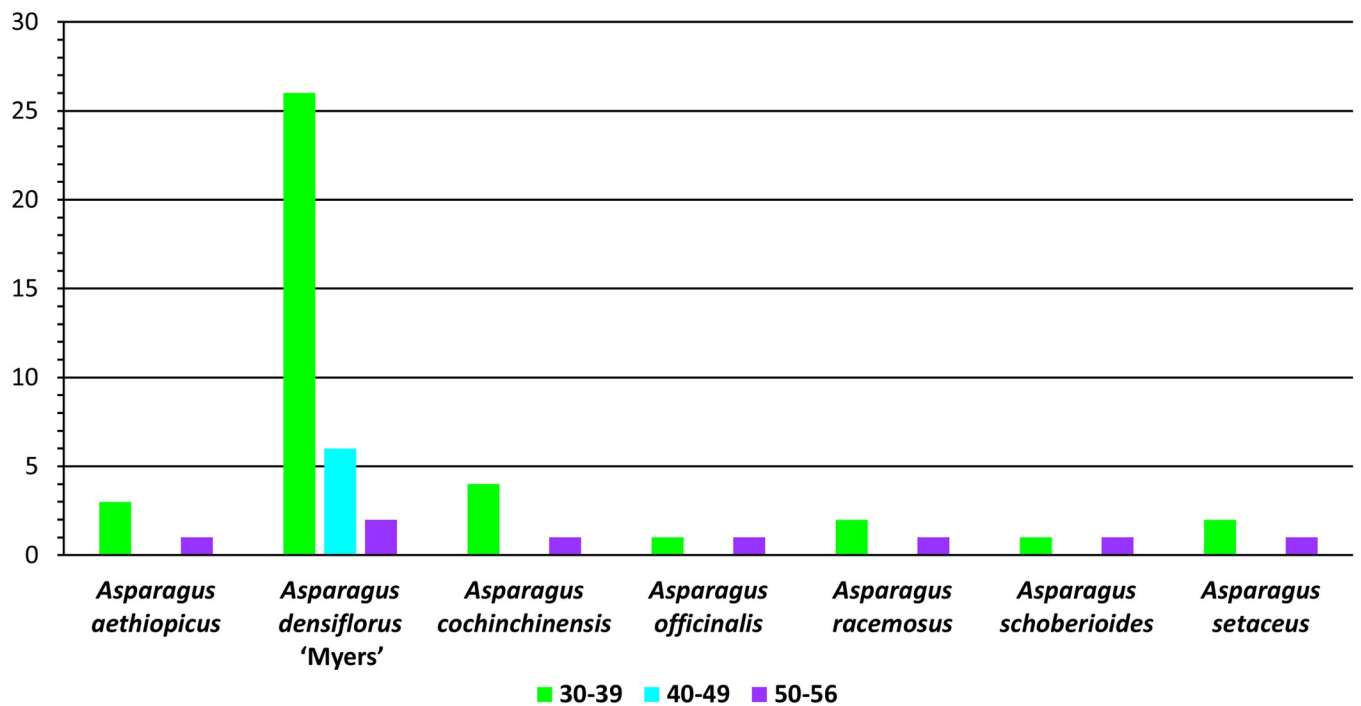


Fig 7. Frequency of long sequence repeats in specified length intervals.

<https://doi.org/10.1371/journal.pone.0266376.g007>

detected. *A. densiflorus* ‘Myers’ had the most in this class, at 26. Each of the three species had at least one 50–56 bp LSR, while *A. densiflorus* ‘Myers’ had two.

Comparative genome analysis

The IR boundaries of the seven genomes were relatively conserved, with some minor variations (contractions and deletions) (Fig 8).

In the LSC/IR_B border, *rpl22* extended into the LSC by 2–5 bp from the junction, for all species except *A. cochinchinensis*, in which it extended it by 24 bp. For *A. officinalis*, *rpl22* was 360 bp long, 3 bp shorter than in the others. *rps19* in the IR_B also exhibited variation, with lengths of 210 bp for *A. aethiopicus*, *A. densiflorus* ‘Myers’, and *A. racemosus*, and 279 bp for the other four species; it extended by 263–332 bp from the LSC/IR_B junction into the IR_B.

The *ycf1* pseudogenes was retained in the border IR_B/SSC for all species, except *A. cochinchinensis* and *A. officinalis*; its length was 912 bp for all species except *A. schoberioides*, in which a 110 bp fragment of the SSC was deleted. *ndhF* in the SSC was 2229 bp long for *A. aethiopicus* and *A. densiflorus* ‘Myers’, and 2223 bp long for the other species; it extended from IR_B/SSC junction by 3 bp for *A. aethiopicus* and *A. densiflorus* ‘Myers’, 7 bp for *A. cochinchinensis*, and 9 bp for the others.

Functional *ycf1* genes (5624–5460 bp long) were located at the SSC/IR_A border for all species except *A. officinalis*, in which an IR_A portion was lost to the SSC, leaving a contracted pseudogene of 3824 bp in length. Further, in *A. setaceus*, the functional *ycf1* extended into the SSC by 307 bp from the SSC/IR_A junction, unlike in the other species.

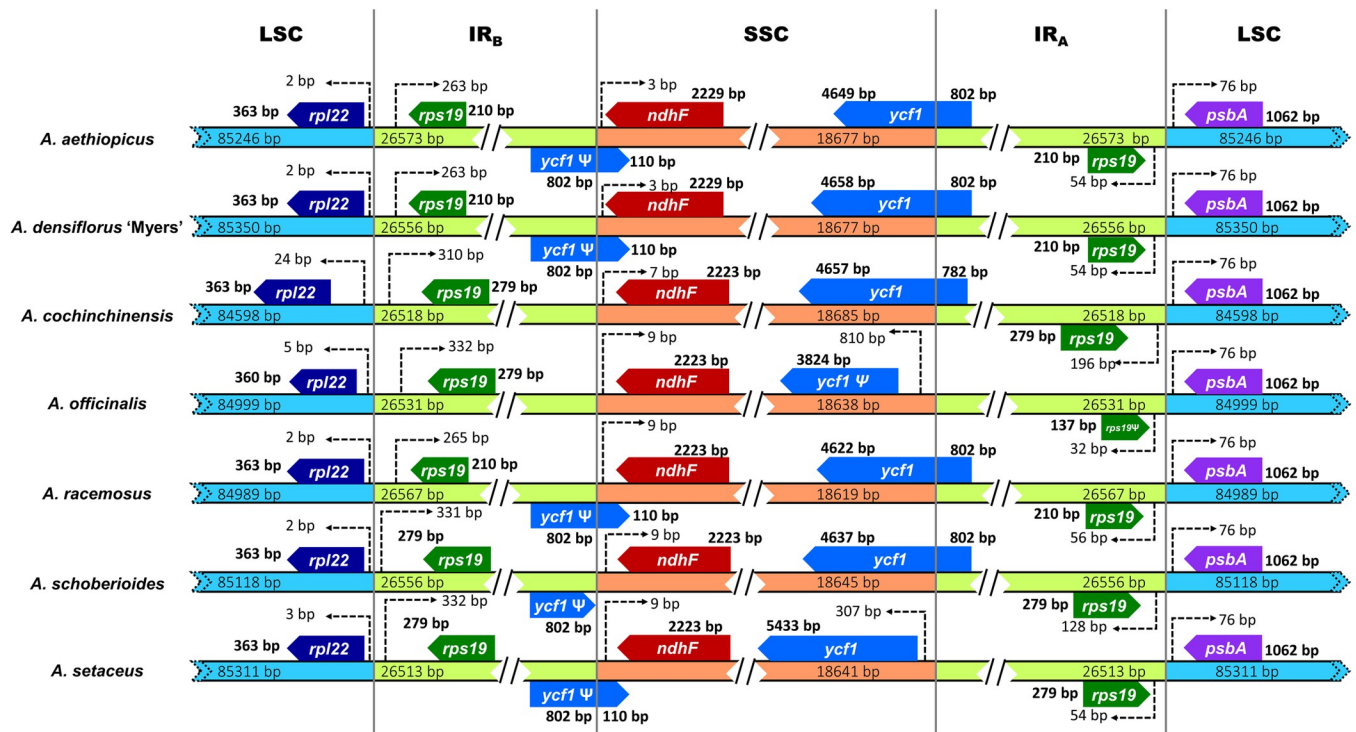


Fig 8. Large single copy (LSC), small single copy (SSC), and inverted repeat (IR) boundary comparison for the seven *Asparagus* cpDNA genomes. Numbers in bold indicate the size of the gene (or gene section) within the specified regions. The numbers next to the dashed arrows indicate distances from the specified junctions. Numbers within the coloured bands indicate the lengths of the respective regions. The direction of gene transcription is presented by the obtuse angles of the pentagons. Ψ, pseudogene. Not to scale.

<https://doi.org/10.1371/journal.pone.0266376.g008>

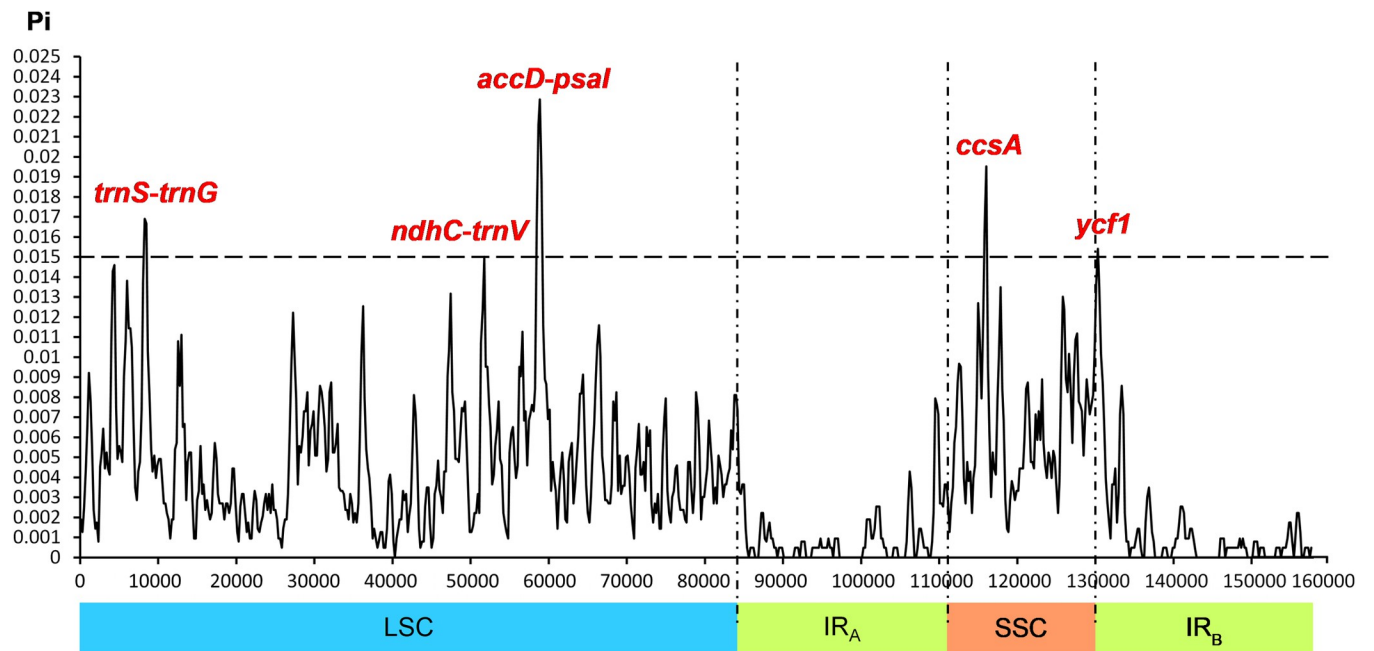


Fig 9. Complete cpDNA genome nucleotide diversity for the seven *Asparagus* species. X-axis: window midpoint; Y-axis: nucleotide diversity value (Pi) for each window. Divergence hotspots ($P_i > 0.015$) are labelled in red above the corresponding position.

<https://doi.org/10.1371/journal.pone.0266376.g009>

At the IR_A/LSC border, *rps19* (137–279 bp long) in IR_A extended by 32–196 bp from the junction, with *A. officinalis* having the shortest extension as a contracted pseudogene.

In the sliding-window analysis, five regions—*trnS-trnG*, *ndhC-trnV*, *accD-psaI*, *ccsA*, and *ycf1*—were identified as divergence hotspots with $P_i \geq 0.015$ (Fig 9). *accD-psaI* was the most variable ($P_i = 0.023$), followed by *ccsA* ($P_i = 0.020$), and *trnS-trnG* ($P_i = 0.017$). These regions represent potential molecular markers for the phylogenetic and population genetics studies of *Asparagus* species. The sequence identity plot, using *A. aethiopicus* as a reference (S2 Fig), revealed different identity level (of <50%) among these five regions between the seven species, with “cracks” among the bars.

Gene order and gene content were highly conserved among the seven species. The sequence identity plot (S2 Fig) revealed highly similar exon (purple) and intron (blue) regions. UTRs (red) in the non-coding regions clearly illustrate the diversity. The average P_i of 0.004 indicates that the sequence diversity of these species is relatively low.

No structural rearrangement was observed. IRs were more conserved than LSCs or SSCs, as illustrated by the high IR similarity in the sequence identity plot and supported by the sliding window analysis. The LSC and SSC regions contained most of the P_i peaks. In contrast, IRs had low nucleotide diversity ($P_i < 0.01$), except for the *ycf1* divergence hotspot at the SSC/IR border. The other four divergence hotspots were within LSCs (*trnS-trnG*, *ndhC-trnV*, and *accD-psaI*) and SSC (*ccsA*).

Phylogenetic analysis

Congeneric relationships in the genus *Asparagus* were examined using three newly assembled cpDNA genomes and four cpDNA genomes from GenBank. ML trees derived from the complete cpDNA genomes, LSC, SSC, and CDS sequences shared the same topology (Fig 10) but different node bootstrap values. *A. setaceus* was sister to the other six *Asparagus* species. The branch containing *A. aethiopicus* and *A. densiflorus* ‘Myers’ had the highest bootstrap value

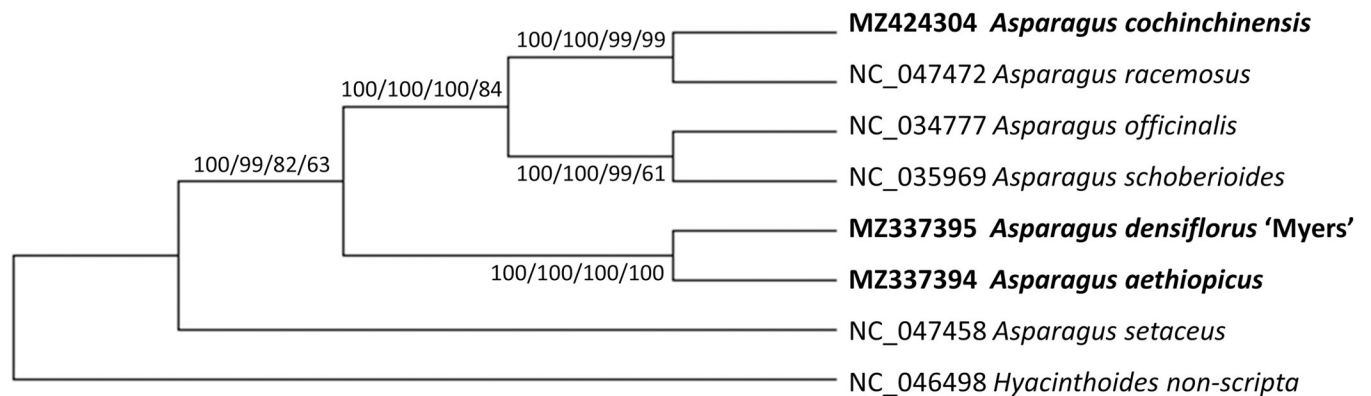


Fig 10. Maximum likelihood (ML) trees based on *Asparagus* cpDNA genomes. Numbers next to the nodes: bootstrap values based on complete cpDNA genomes/LSC/SSC/CDS sequences. The topologies are identical. Bold taxa: the three newly assembled cpDNA genomes.

<https://doi.org/10.1371/journal.pone.0266376.g010>

(100) in all four ML trees, supporting the close relationship between these two species. *A. cochinchinensis* and *A. racemosus* formed a sister clade to *A. officinalis* and *A. schoberioides* (bootstrap values of 100 for complete cpDNA genomes, LSC, and SSC, and 84 for CDS). The close relationship between *A. cochinchinensis* and *A. racemosus* was well supported (bootstrap values of 100 for complete cpDNA genomes and LSC, and 99 for SSC and CDS). This new grouping differs from both traditional taxonomical classifications and molecular phylogenies [5, 6, 11]. We expected *A. racemosus*, a monoecious species, to group with the three other monoecious species from South Africa. Instead, it was nested within the group of dioecious and Eurasian species in the ML trees, with high bootstrap values.

The ML tree based on IR sequences also exhibited unexpected grouping (Fig 11): *A. racemosus* was still nested with the dioecious species, which were sister to *A. officinalis* and *A. schoberioides*, with moderate support (bootstrap value = 71).

The close relationship between *A. aethiopicus* and *A. densiflorus* 'Myers' was supported by the ML trees based on complete cpDNA genomes, LSC, SSC, and CDS sequences (Fig 10) and was further validated by the IR-based tree, with bootstrap values of 100.

Discussion

Molecular insights for nomenclatural confusion

A. aethiopicus and *A. densiflorus* 'Myers' are nomenclaturally controversial. Batchelor and Scott (2006) [67] questioned the taxonomic identity of the cultivar 'Myers' (foxtail asparagus),

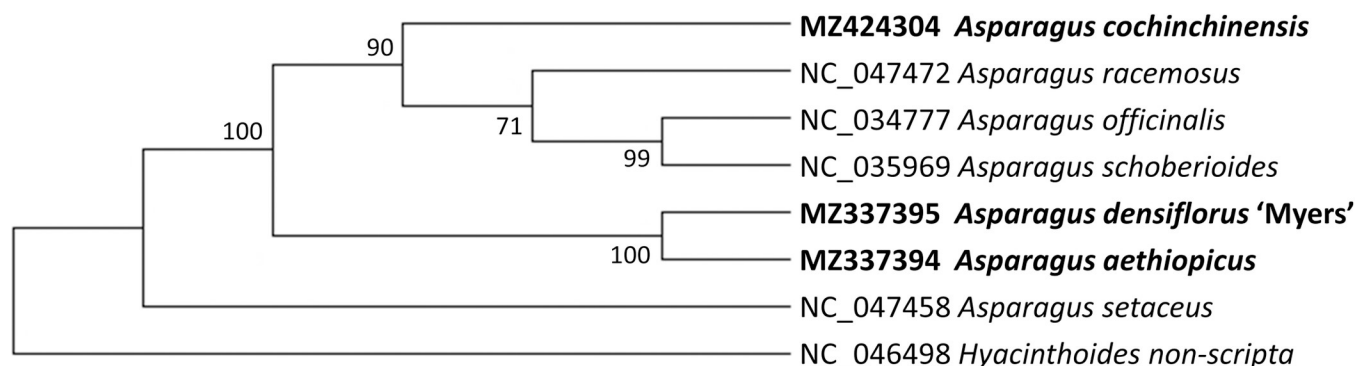


Fig 11. Maximum likelihood (ML) trees based on inverted repeats (IRs) for *Asparagus*. Numbers next to the nodes: bootstrap values on IR_A and IR_B. Bold taxa: the three newly assembled cpDNA genomes.

<https://doi.org/10.1371/journal.pone.0266376.g011>

which is often recorded as a cultivar of *A. densiflorus* [1, 3, 5, 11, 15, 51, 57, 64, 67, 80, 83]. In contrast, some have suggested placing the *Asparagus* cultivars ‘Sprengeri’ and ‘Myers’ under *A. aethiopicus* [4, 67, 76, 77]. The Royal Botanic Gardens Victoria [78, 108] has adopted the name *A. aethiopicus* ‘Myersii’ for foxtail asparagus.

A. densiflorus and *A. aethiopicus* differ primarily in their growth habit, with the former not a climber and rarely over 1 m tall and the latter an erect herb of 1 m or more and climbing up to 7 m [4, 76]. From our observations, the *Asparagus* cultivar ‘Myers’ never climbs, even when it is not pot-bound. This growth habit does not correspond with the circumscription of *A. aethiopicus* emphasised by Green (1986) [76] and Judd (2001) [4]. We agree with Batchelor and Scott (2006) that foxtail asparagus should be considered a cultivar of *A. densiflorus* [67], and hence the legitimate name should be *Asparagus densiflorus* (Kunth) Jessop ‘Myers’.

Our findings show that *A. aethiopicus* and *A. densiflorus* ‘Myers’ are phylogenetically close, despite their morphological and growth habit differences, with bootstrap values of up to 100 for ML trees based on complete cpDNA genomes, LSC, SSC, IR, or CDS (Figs 10 and 11). Their gene numbers, GC content (Table 2), genome structure (Fig 2), and IR border (Fig 8) are similar. This supports the traditional classifications, which consistently place them under the same generic circumscription: genus *Asparagopsis* [71], genus *Asparagus* section *Falcati* [8], genus *Asparagus* section *Racemosi* [15], or genus *Protasparagus* [16] (S1 Fig and S1 Table). Using short-length DNA regions, Norup *et al.* [6] suggested placing the two species in an *Asparagus*–*Racemose* clade–*Racemose* 1 clade. Our phylogenetic results, which group the cpDNA genomes of *A. aethiopicus* and *A. densiflorus* ‘Myers’, are consistent with this.

The two species showed minor differences. In terms of LSR number and type, *A. densiflorus* ‘Myers’ differed significantly from *A. aethiopicus* and the other species. The cpDNA genome of *A. densiflorus* ‘Myers’ had the most LSRs, and this was the only species with reverse repeats and 40–49 bp LSRs (Figs 6 and 7). SSRs have been used to identify cultivars of potatoes [109, 110], apples [111], and sunflowers [112]. However, these *Asparagus* species did not differ significantly in SSRs. Nonetheless, the distinctive LSR patterns of *A. densiflorus* ‘Myers’ could provide a molecular authentication marker.

Our phylogenetic analysis revealed the close relationship between *A. aethiopicus* and *A. densiflorus* ‘Myers’ but did not elucidate the species origin of the cultivar. According to Article 21.1 of ICNCP, “The name of a cultivar is a combination of the correct name of the genus or lower taxon to which it is assigned under the ICN, or its unambiguous common name, with a cultivar epithet” [86]. We suggest two treatments to clarify *A. densiflorus* ‘Myers’ nomenclature: first, to combine only the genus name with the cultivar epithet, as *Asparagus* ‘Myers’, since this cultivar epithet has not been used for other cultivars being assigned to other *Asparagus* species; second, to combine the common name and the cultivar epithet, as asparagus ‘Myers’, since the common name of the genus *Asparagus* is unambiguous and is identical to the genus name.

Unexpected placement of *A. racemosus*

Taxonomists have attempted to divide the genus *Asparagus* into three major groups. The first, characterised by flattened and leaf-like cladodes, basally connate perianth segments, and filaments connated into tubes, was classified as the genus *Myrsiphyllum* by Willdenow (1808) [14], Kunth (1850) [71], and Obermeyer (1984) [17], and as the genus *Asparagus* subgenus *Myrsiphyllum* by Baker (1875) [7]. The second and third groups comprise the species with filiform to linear cladodes: the second, comprising monoecious and African species with free perianth segments and filaments, was classified as the genus *Asparagopsis* by Kunth (1850) [71], the genus *Asparagus* subgenus *Asparagopsis* by Baker (1875) [7], and the genus *Protasparagus* by Obermeyer (1983) [17]; the third, comprising dioecious and Eurasian species with

basally connate perianth segments, was classified as the genus *Asparagus* by Kunth (1850) [71] and the genus *Asparagus* subgenus *Euasparagus* by Baker (1875) [7].

A. racemosus, a monoecious species widespread throughout Africa, Asia, and Australia [10, 113], has traditionally been classified into the second group. Fukuda *et al.* [5] and Kubota *et al.* [11] placed *A. racemosus* in genus *Asparagus* subgenus *Protasparagus*, whereas Norup *et al.* [6] placed it in the *Asparagus*–*Racemose* clade–*Racemose* 2 clade.

We expected *A. racemosus* to cluster with its relatives in the same group, i.e. *A. aethiopicus*, *A. densiflorus* ‘Myers’, and *A. setaceus*. However, one *A. racemosus* specimens (NC_047472) unexpectedly clustered with the dioecious species *A. cochinchinensis*, *A. officinalis*, and *A. schoberioides* in the ML trees (Figs 9 and 10), using both complete cpDNA genomes and sequence portions. This is contrary to Lee *et al.* (1997) [114] who, using restriction fragment length polymorphism cpDNA analysis, showed that no monoecious species were clustered within the monophyletic group of dioecious species (*A. officinalis*, *A. schoberioides*, or *A. cochinchinensis*) [114].

Short cpDNA regions of *A. racemosus* (ca. 300–1000 bp) were reported by Fukuda *et al.* (*petB* intron and *petD-rpoA*) [5], Kubota *et al.* (*rpl32-trnL*, *trnQ-5'rps16*, *ndhF-rpl32*, *psbD-trnT*, *3'rps16-5'trnK*) [11], and Norup *et al.* (*3' ndhF*, *psbA-trnH*, *trnD-trnT*) [6]. We attempted to determine the start and stop positions of these regions in NC_047472. Ten extracted sequences of the corresponding length (S2 Table) were screened using the NCBI Basic Local Alignment Search Tool, and only *trnQ-rps16* (sequence identity 98.70%), *psbA-trnH* (97.11% and 96.84%), *rpl32-trnL* (96.49%), *petD-rpoA* (96.86%), and *trnD-trnT* (97.71%) matched the respective regions of *A. racemosus*. GenBank did not contain any voucher information for NC_047472. Because of this lack of voucher information, we are unable to further verify this unanticipated and unlikely grouping. Our intra-generic analyses were constrained by the limited sample size. Further studies on *A. racemosus* phylogeny are recommended.

Conclusion

Complete cpDNA genomes of three *Asparagus* specimens collected in Hong Kong were *de novo* assembled, annotated, and compared with those of congeners. The seven genomes were relatively conserved in terms of gene content, gene order, and genome structure. *A. densiflorus* ‘Myers’ differed significantly from the others in LSR number and type. Five divergence hotspots were identified in the sliding-window analysis ($P_i \geq 0.015$). Our phylogenetic analysis elucidates the generic subdivision and the nomenclatural complexity of *A. aethiopicus* and *A. densiflorus* ‘Myers’. The novel placement of *A. racemosus*, contrary to previous morphological and molecular classifications, requires further verification. We suggest two ICNCP-compliant names for *A. densiflorus* ‘Myers’, namely *Asparagus* ‘Myers’ and *asparagus* ‘Myers’. These *de novo* assembled cpDNA genomes provide potential genomic resources, elucidating *Asparagus* taxonomy, application, and conservation.

Supporting information

S1 Fig. The historical changes on the generic subdivision of the genus *Asparagus*.
(PDF)

S2 Fig. Visualisation of the alignments of 7 *Asparagus* chloroplast genomes using *A. aethiopicus* as a reference.
(PDF)

S3 Fig. Specimen photos of voucher K. H. Wong 092, 107, and 109.
(PDF)

S1 Table. The historical changes on taxonomical status of the 7 studied *Asparagus* species. (XLSX)

S2 Table. Extracted sequences from cpDNA of *Asparagus racemosus* (NC_047472.1). (XLSX)

Author Contributions

Conceptualization: Kwan-Ho Wong.

Data curation: Kwan-Ho Wong, Bobby Lim-Ho Kong.

Formal analysis: Kwan-Ho Wong.

Funding acquisition: Pang-Chui Shaw, David Tai-Wai Lau.

Investigation: Kwan-Ho Wong.

Methodology: Bobby Lim-Ho Kong.

Project administration: Pang-Chui Shaw, David Tai-Wai Lau.

Resources: Pang-Chui Shaw, David Tai-Wai Lau.

Software: Bobby Lim-Ho Kong.

Supervision: Pang-Chui Shaw, David Tai-Wai Lau.

Validation: Bobby Lim-Ho Kong, Tin-Yan Siu, Hoi-Yan Wu, David Tai-Wai Lau.

Visualization: Kwan-Ho Wong.

Writing – original draft: Kwan-Ho Wong.

Writing – review & editing: Tin-Yan Siu, Hoi-Yan Wu, Grace Wing-Chiu But.

References

1. Bailey LH, Bailey EZ, Staff of L. H. Bailey Hortorium, Cornell University. Hortus Third: A Concise Dictionary of Plants Cultivated in the United States and Canada. New York: Macmillan Publishing Company & London: Collier Macmillan Publisher; 1976.
2. Dahlgren RMT, Clifford HT, Yeo PF, Faden RB, Jacobsen N, Jacobsen K, et al. The Families of the Monocotyledons: Structure, Evolution, and Taxonomy. Berlin, Heidelberg, New York, Tokyo: Springer-Verlag; 1985. ISBN: 3-540-13655-X
3. The Royal Horticultural Society (RHS). The New Royal Horticultural Society Dictionary of Gardening. London: The Macmillan Press Limited & New York: The Stockton Press; 1992. ISBN: 1-56159-001-0.
4. Judd W. The Asparagaceae in the Southeastern United States. Harv Pap Bot. 2001; 6(1):223–244. Available from: <http://www.jstor.org/stable/41761647>
5. Fukuda T, Ashizawa H, Suzuki R, Ochiai T, Nakamura T, Kanno A, et al. (2005). Molecular phylogeny of the genus *Asparagus* (Asparagaceae) inferred from plastid petB intron and petD-rpoA intergenic spacer sequences. Plant Species Biol. 2005; 20(2):121–132. <https://doi.org/10.1111/j.1442-1984.2005.00131.x>
6. Norup MF, Petersen G, Burrows S, Bouchenak-Khelladi Y, Leebens-Mack J, Pires JC, et al. Evolution of *Asparagus* L. (Asparagaceae): Out-of-South-Africa and multiple origins of sexual dimorphism. Mol Phylogenet Evol. 2015; 92:25–44. <https://doi.org/10.1016/j.ympev.2015.06.002> PMID: 26079131
7. Baker JG. Tribus VIII. Asparageae, Revision of the Genera and Species of Asparagaceae. Bot J Linn Soc. 1875; 594–692. Available from: <https://www.biodiversitylibrary.org/item/8365>
8. Baker JG. II. *Asparagus*, Linn. In: Thiselton-Dyer WT, editor. Flora capensis: being a systematic description of the plants of the Cape colony, Caffraria, & Port Natal. Volume 6. Kent: L. Reeve & Co., Ltd; 1896. pp. 256–274. <https://doi.org/10.5962/bhl.title.821>
9. Greiner T. *Asparagus*. In: Bailey LH, editor. The Standard Cyclopaedia of Horticulture. New York: The Macmillan Company; 1919. pp. 406–411. <https://doi.org/10.5962/bhl.title.23351>

10. Clifford HT, Conran JG. Liliaceae. In: George AS, Purdie RW, editors. Flora of Australia. Volume 45. Canberra: Australian Government Publishing Service; 1987. pp. 148–419. Available from: <https://www.environment.gov.au/system/files/pages/34b53009-bdf7-4c4d-93a6-4a9ab3c14cf7/files/flora-australia-45-hydatellaceae-liliaceae.pdf>
11. Kubota S, Konno I, Kanno A. Molecular phylogeny of the genus *Asparagus* (Asparagaceae) explains interspecific crossability between the garden asparagus (*A. officinalis*) and other *Asparagus* species. Theor Appl Genet. 2012; 124(2):345–354. <https://doi.org/10.1007/s00122-011-1709-2> PMID: 21947345
12. Arber A. *Myrsiphyllum* and *Asparagus*: a Morphological Study. Ann Bot. 1924; 38(152):635–659. Available from: <http://www.jstor.org/stable/43237250>
13. Arber A. *Monocotyledons: A Morphological Study*. Cambridge: Cambridge University Press; 1925. <https://doi.org/10.1017/CBO9780511708626>
14. Willdenow CL. (1808). Nähere Bestimmung einiger Liliengewächse, besonders aber derjenigen, die zur sechsten Klasse und dritten Ordnung des Linnischen Systems gehören. In: Janrgane Z, editor. Der Gesellschaft Naturforschender Freunde zu Berlin Magazin für die neuesten Entdeckungen in der gesammten Naturkunde. Berlin: Realschulbuchhandlung; 1808. pp. 14–30. Available from: http://resolver.sub.uni-goettingen.de/purl?PPN608227714_0002
15. Jessop JP. The Genus *Asparagus* in South Africa. Bothalia. 1966; 9(1):31–96. <https://doi.org/10.4102/abc.v9i1.1576>
16. Obermeyer AA. *Protasparagus* Oberm. nom. nov.: new combinations. S Afr J Bot. 1983; 2(3):243–244. Available from: <https://cyberleninka.org/article/n/676191/viewer>
17. Obermeyer AA. Revision of the genus *Myrsiphyllum* Willd. Bothalia. 1984; 15(1&2):77–88. <https://doi.org/10.4102/abc.v15i1/2.1106>
18. Altunel TA. Morphological and Habitat Characteristics of *Asparagus* (*Asparagus officinalis* L.) and Socio-Economic Structure of Producers. Turk Tarim Gida Bilim Teknol Derg. 2021; 9(6):1092–1099. <https://doi.org/10.24925/turjaf.v9i6.1092-1099.4269>
19. Bopana N, Saxena S. In vitro propagation of a high value medicinal plant: *Asparagus racemosus* Willd. In Vitro Cellular & Developmental Biology-Plant. 2008; 44(6):525–532. <https://doi.org/10.1007/s11627-008-9137-y>
20. Quisumbing E. *Medicinal plants of the Philippines*. Manila: Bureau of Printing; 1951. PMID: 14874206
21. Watt JM, Breyer-Brandwijk MG. *The medicinal and poisonous plants of southern and eastern Africa*. Edinburgh: E. & S. Livingstone Ltd; 1962.
22. Lewis WH, Elvin-Lewis MPF. *Medical Botany: Plants Affecting Human Health*. New York: John Wiley & Sons; 1977.
23. Perry LM, Metzger J. *Medicinal plants of East and Southeast Asia: attributed properties and uses*. Cambridge: MIT Press; 1980. ISBN: 9780262160766.
24. Duke JA, Ayensu EA. *Medical plants of China. Volume 2*. Algonac: Reference Publication; 1985.
25. Kapoor LD. *CRC Handbook of Ayurvedic Medicinal Plants*. Boca Raton: CRC Press; 1990. ISBN: 9781315891897.
26. Chevallier A. *The Encyclopedia of Medicinal Plants*. New York: Dorling Kindersley Publishing; 1996. ISBN: 9780789410672.
27. The Royal Horticultural Society (RHS). *The New Encyclopedia of Herbs & Their Uses*. 3rd ed. London: Dorling Kindersley Limited; 2008. ISBN: 978-1-4053-3238-5.
28. Leon C, Lin LY. *Chinese Medicinal Plants, Herbal Drugs and Substitutes: an identification guide*. Kew: Royal Botanic Garden, Kew; 2017. ISBN: 978-1-84246-387-1.
29. Jahan R, Jannat K, Shoma JF, Khan MA, Shekhar HU, Rahmatullah M. Drug Discovery and Herbal Drug Development: A Special Focus on the Anti-diarrheal Plants of Bangladesh. In: Sen S, Chakraborty R, editors. *Herbal Medicine in India*. Singapore: Springer; 2020. https://doi.org/10.1007/978-981-13-7248-3_23
30. The State Pharmacopoeia Commission of the People's Republic of China. *Pharmacopoeia of the People's Republic of China 2010. Volume 1*. Beijing: China Medical Science Press; 2010. ISBN: 978-7-5067-5013-4.
31. The State Pharmacopoeia Commission of the People's Republic of China. *Pharmacopoeia of the People's Republic of China 2020, Volume 1*. [Written in Chinese]. Beijing: China Medical Science Press; 2020. ISBN: 978-7-5214-1574-2.
32. Jiangsu New Medical College. *Encyclopedia of Chinese Materia Medica. Volume 1*. Shanghai: Shanghai People's Press; 1977. pp. 858–861.

33. Fan LL, Zhu S, Chen HB, Yang DH, Cai SQ, Komatsu K. Molecular analysis of *Stemona* plants in China based on sequences of four chloroplast DNA regions. *Biol Pharm Bull*. 2009; 32(8):1439–1446. <https://doi.org/10.1248/bpb.32.1439> PMID: 19652387
34. But P, Shaw P, Lin G, Jiang R, Xu Y. Authentication and Quality Assessment of the Antitussive Herb Baibu (*Radix Stemona*). *Adv Bot Re*. 2012; 62: 1–33. <https://doi.org/10.1016/B978-0-12-394591-4.00001-5>
35. Fu H, Wang JP, Zhu L, Cai L, Yang MH. Studies on the chemical constituents of *Asparagus filicinus* produced in Yunnan. *Chemical Research and Application*. 2020; 32(4): 637–644.
36. Hu JY, Lu Y, Huang YF, Chen DF. Determination of Total Saponins of *Asparagus filicinus* Root by Colorimetry and its Quality Standard. *Shizhen Guo Yi Guo Yao*. 2010; 21(2): 307–308.
37. Hu SY. *Food Plants of China*. Hong Kong: The Chinese University Press; 2005. ISBN: 962-201-860-2.
38. Geoffriau E, Denoue D, Rameau C. Assessment of genetic variation among asparagus (*Asparagus officinalis* L.) populations and cultivars: agromorphological and isozymic data. *Euphytica*. 1992; 61:169–179. <https://doi.org/10.1007/BF00039655>
39. Mercati F, Riccardi P, Harkess A. Single nucleotide polymorphism-based parentage analysis and population structure in garden asparagus, a worldwide genetic stock classification. *Mol Breeding*. 2015; 35(59):1–12. <https://doi.org/10.1007/s11032-015-0217-5>
40. Moreno R, Espejo JA, Cabrera A, Millán T, Gil J. Ploidic and Molecular Analysis of 'Morado de Huetor' asparagus (*Asparagus officinalis* L.) population; a Spanish tetraploid landrace. 2006; 53:729–736. *Genet Resour Crop Evol*. <https://doi.org/10.1007/s10722-004-4717-0>
41. Stephen CT, Elmer WH. An in vitro assay to evaluate sources of resistance in *Asparagus* spp. to *Fusarium* crown and root rot. *Plant Dis*. 1988; 72(4):334–337. Available from: https://www.apsnet.org/publications/plantdisease/backissues/Documents/1988Articles/PlantDisease72n04_334.PDF.
42. Stephen CT, Vries RM, Sink KC. Evaluation of *Asparagus* species for resistance to *Fusarium oxysporum* f. sp. *asparagi* and *F. moniliforme*. *HortScience*. 1989; 24:365–368.
43. Kanh RP, Anderson HW, Hepler PR, Linn MB. An Investigation of asparagus rust in Illinois: its causal agent and its control. In: Bulletin 559, University of Illinois Agricultural Experiment Station. Illinois: University of Illinois Agricultural Experiment Station; 1952. pp. 1–56. Available from: <https://archive.org/details/investigationofa00unse/mode/2up>
44. Norton JB. Methods used in breeding *Asparagus* for rust resistance. In: Bulletin no. 263, Bureau of Plant Industry, U. S. Department of Agricultural Science. Washington: Government Printing Office; 1913. pp. 9–60. Available from: <https://babel.hathitrust.org/cgi/pt?id=loc.ark:/13960/t2q532z8z&view=1up&seq=7>
45. Gonzalez Castanon ML, Falavigna A. *Asparagus* germplasm and interspecific hybridization. *Acta Hort*. 2008; 776:319–326. <https://doi.org/10.17660/ActaHortic.2008.776.41>
46. Falavigna A, Alberti P, Casali PE, Toppino L, Huaisong W, Mennella G. Interspecific hybridization for *Asparagus* breeding in Italy. *Acta Hort*. 2008; 776:291–297. <https://doi.org/10.17660/ActaHortic.2008.776.37>
47. Bansal RK, Menzies SA, Broadhurst PG. Screening of *Asparagus* species for resistance to *Stemphylium* leaf spot. *New Zealand Journal of Agricultural Research*. 1986; 29(3):539–545. <https://doi.org/10.1080/00288233.1986.10423507>
48. Sonoda T, Uragami A, Itoh K, Kohmura H, Ohwada M, Kaji K. Evaluation of *Asparagus* species and comparison between sexes in *A. officinalis* cultivars for resistance to stem blight. *Journal of the Japanese Society for Horticultural Science*. 2001; 70(2):244–250. <https://doi.org/10.2503/jshs.70.244>
49. Kanno A, Yokoyama J. Chapter 3 *Asparagus*. In: Kole C, editor. *Wild Crop Relatives: Genomic and Breeding Resources Vegetables*. Heidelberg, Dordrecht, London, New York: Springer; 2011. pp. 23–42. <https://doi.org/10.1007/978-3-642-20450-0>
50. Ito T, Ochiai T, Ashizawa H, Shimodate T, Sonoda T, Fukuda T, et al. Production and analysis of reciprocal hybrids between *Asparagus officinalis* L. and *A. schoberioides* Kunth. *Genet Resour Crop Evol*. 2007; 54:1063–1071. <https://doi.org/10.1007/s10722-006-9186-1>
51. McCollum GD. *Asparagus densiflorus* cultivars Sprenger and Myers cross-pollinations with *A. officinalis* and other species. *Asparagus Newsletter*. 1988; 6:1–10.
52. Marcella'n ON, Camadro EL. Formation and development of embryo and endosperm in intra- and inter-specific cross of *Asparagus officinalis* and *A. densiflorus* cv. Sprenger. *Sci Hort*. 1999; 81:1–11. [https://doi.org/10.1016/S0304-4238\(98\)00255-6](https://doi.org/10.1016/S0304-4238(98)00255-6)
53. Marcella'n ON, Camadro EL. Self- and cross-incompatibility in *Asparagus officinalis* and *Asparagus densiflorus* cv. Sprenger. *Can J Bot*. 1996; 74(10):1621–1625. <https://doi.org/10.1139/b96-196>

54. Ochiai T, Sonoda T, Kanno A, Kameya T. Interspecific hybrids between *Asparagus schoberioides* Kunth and *A. officinalis* L. *Acta Hort.* 2002; 589:225–229. <https://doi.org/10.17660/ActaHortic.2002.589.31>
55. Tardio J, Padro-de-Santayana M, Morales R. Ethnobotanical review of wild edible plants in Spain. *Bot J Linn Soc.* 2006; 152:27–71. <https://doi.org/10.1111/j.1095-8339.2006.00549.x>
56. Peters CR, O'Brien EM, Drummond RB. Asparagaceae. In: *Edible wild plants of Sub-Saharan Africa*. Kew: Royal Botanic Gardens, Kew; 1992. pp. 13.
57. Walters SM, Cullen J, Brady A, Brickell CD, Green PS, Lewis J, et al. *The European Garden Flora*. Volume 1. 1st ed. Cambridge: Cambridge University Press; 1986. ISBN: 0-521-24859-0 (hardback).
58. South Florida Water Management District. *WaterWise: South Florida Landscapes*. Florida: Water Management District; 2003 [cited 2021 Sep 15]. Available from: https://www.sfwmd.gov/sites/default/files/documents/ww0_waterwise_all.pdf.
59. Florida Exotic Pest Plant Council (FLEPPC). *Florida Exotic Pest Plant Council's 2019 List of Invasive Plant Species*. Florida: Florida Exotic Pest Plant Council; 2019 [cited 2021 Sep 15]. Available from: http://bugwoodcloud.org/CDN/fleppc/plantlists/2019/2019_Plant_List_ABSOLUTE_FINAL.pdf
60. Wong M. *Xeriscape Plants. Ornamentals and Flowers*, Cooperative Extension Service, College of Tropical Agriculture and Human Resources, University of Hawai'i at Mānoa. 2008; OF-42: 1–7 [cited 2021 Sep 15]. Available from: <https://www.ctahr.hawaii.edu/oc/freepubs/pdf/OFF-42.pdf>
61. Development Bureau, The Government of Hong Kong Special Administrative Region. *Asparagus cochinchinensis*. In: *Skyrise Greenery—Pictorial Guide to Plant Resources for Skyrise Greenery in Hong Kong*; 2016 [cited 2021 Aug 19]. Available from: https://www.greening.gov.hk/filemanager/content/pdf/green_technologies/guide/61_Aspargus_cochinchinensis.pdf.
62. Development Bureau, The Government of Hong Kong Special Administrative Region. *Asparagus densiflorus* cv. *Sprengeri*. In: *Skyrise Greenery—Pictorial Guide to Plant Resources for Skyrise Greenery in Hong Kong*; 2016 [cited 2021 Aug 19]. Available from: https://www.greening.gov.hk/filemanager/content/pdf/green_technologies/guide/61_Aspargus_cochinchinensis.pdf.
63. Development Bureau, The Government of Hong Kong Special Administrative Region. *Asparagus densiflorus* 'Myersii'. In: *Skyrise Greenery—Pictorial Guide to Plant Resources for Skyrise Greenery in Hong Kong*; 2016 [cited 2021 Aug 19]. Available from: https://www.greening.gov.hk/filemanager/content/pdf/green_technologies/guide/61_Aspargus_cochinchinensis.pdf.
64. Staples G, Herbst D. *A Tropical Garden Flora: plants cultivated in the Hawaiian Islands and other tropical places*. Hawai'i: Bishop Museum Press; 2005. ISBN: 1-58178-039-7.
65. Conran JG, Forster PI. *Protasparagus africanus* (Asparagaceae) a serious weed for south-eastern Queensland. *Austrobaileya*. 1986; 2(3):300–304. Available from: <https://www.jstor.org/stable/41738686>.
66. Bowden D, Rogers RW. *Protasparagus densiflorus*: an environmental weed of coastal vegetation reverses. *Pac Conserv Biol*. 1995; 2(3):293–298. <https://doi.org/10.1071/PC960293>
67. Batchelor KL, Scott JK. Review of the current taxonomic status and authorship for *Asparagus* weeds in Australia. *Plant Prot Q*. 2006; 21(3):128–130. Available from: <https://caws.org.nz/PPQ202122/PPQ%2021-3%20pp128-130%20Batchelor.pdf>.
68. Linnaeus C. *Species Plantarum*. Tomus I. Holmiae: Impensis Laurentii Salvii; 1753. <https://doi.org/10.5962/bhl.title.669>
69. Malcomber ST, Demissew S. The Status of *Protasparagus* and *Myrsiphyllum* in the Asparagaceae. *Kew Bull.* 1993; 48(1):63–78. <https://doi.org/10.2307/4115749>
70. Fellingham AC, Meyer NL. New combinations and a complete list of *Asparagus* species in southern Africa (Asparagaceae). *Bothalia*. 1995; 25(2):205–209. <https://doi.org/10.4102/abc.v25i2.728>
71. Kunth CS. *Enumeratio Plantarum Omnium Hucusque Cognitarum, Secundum Familias Naturales Disposita, Adjectis Characteribus, Differentiis, et Synonymis*. Tomus Quintus. Stutgardiae et Tubingae: Sumtibus JG Collae; 1850. Available from: <https://bibdigital.rjb.csic.es/records/item/11045-redirect>.
72. Mathew B. Notes on Tropical African Asparagaceae. *Kew Bull.* 1989; 44(1):181–182. <https://doi.org/10.2307/4114662>
73. Huber H. Die Samenmerkmale und Verwandtschaftsverhältnisse der Liliifloren. In: *Mitteilungen der Botanischen Staatssammlung*. Band VIII. München: H. Merxmüller; 1969. pp. 219–538. Available from: <https://www.biodiversitylibrary.org/page/15148674#page/639/mode/1up>.
74. Regel E. *Liliaceae Asparageae*. In: Regel E, editor. *Descriptiones Plantarum Nonnularum Horti Imperialis Botanici Petropolitani In Statu Vivo Examinatarum*. Acta Horti Petropolitani. 1890; 11(8):302. Available from: <https://www.biodiversitylibrary.org/item/53023>.

75. Regel E. *Asparagus Sprenger* Rgl. In: Gartenflora. Berlin: Verlag von Paul Parey; 1890. pp. 490–492. Available from: <https://www.biodiversitylibrary.org/item/134139>.
76. Green PS. The correct name for *Asparagus sprengeri*. The Plantsman. 1986; 7:249–250.
77. Straley GB, Utech FH. *Asparagus aethiopicus* Linnaeus. In: Flora of North America Editorial Committee, editors. Flora of North America North of Mexico [Internet]. Volume 26. New York, Oxford: Oxford University Press; 2003. pp. 213–214 [cited 10 Sep 2021]. Available from: http://floranorthamerica.org/Asparagus_aethiopicus.
78. Conran J. Family Asparagaceae. In: Spencer R, editor. Horticultural Flora of South-eastern Australia: The identification of Garden and Cultivated Plants. Volume 5. Flower Plants: Monocotyledons. Sydney: University of New South Wales Press Ltd; 2005. pp. 284–286. ISBN: 0868408328.
79. Hu QM. 10. *Asparagus* L. In: Hong Kong Herbarium & South China Botanical Garden, editors. Flora of Hong Kong. Volume 4. Hong Kong: Agriculture, Fisheries and Conservation Department; 2011. pp. 266–267. ISBN: 978-988-98253-9-3.
80. The Royal Horticultural Society (RHS). The New Encyclopedia of Plants and Flowers. 3rd ed. London: Dorling Kindersley Limited; 1999. ISBN: 0-7513-0800-5.
81. The Royal Horticultural Society (RHS). The Award of Garden Merit lists—Ornamentals; 2021 Jul [cited 2021 Aug 20]. Available from: <https://www.rhs.org.uk/plants/pdfs/agm-lists/agm-ornamentals.pdf>.
82. Obermeyer AA, Immelamn KL, Bos JJ. (1992). Asparagaceae. In: Leistner OA, du Plessis E, editors. Flora of Southern Africa. Volume 5, Part 3. Dracaenaceae, Asparagaceae, Luzuriagaceae and Smilacaceae. Pretoria: National Botanical Institute; 1992. pp. 11–82.
83. Huttleston DG. The names of three commonly cultivated ornamental asparagus [Liliaceae]. Baileya. 1970; 17:58–59.
84. Council of Heads of Australasian Herbaria (CHAH). *Asparagus densiflorus* 'Meyers'; 2006 [cited 2021 Sep 15]. Vascular Plants National Species List Services [Internet]. Available from: <https://id.biodiversity.org.au/instance/apni/859654>.
85. Saha PS, Ray S, Sengupta M, Jha S. Molecular phylogenetic studies based on rDNA ITS, cpDNA trnL intron sequence and cladode characteristics in nine *Protasparagus* taxa. Protoplasma. 2015; 252: 1121–1134. <https://doi.org/10.1007/s00709-014-0746-5> PMID: 25534258
86. International Society for Horticultural Science. International Code of Nomenclature for Cultivated Plants (ICNCP). 9th ed. Scripta Horticulturae. 2016; 18:1–190. ISBN:978-94-6261-116-0. Available from: https://www.ishs.org/sites/default/files/static/ScriptaHorticulturae_18.pdf.
87. Li Y, Zhang Z, Yang J, Lv G. Complete chloroplast genome of seven *Fritillaria* species, variable DNA markers identification and phylogenetic relationships within the genus. PLoS ONE. 2018; 13(3): e0194613. <https://doi.org/10.1371/journal.pone.0194613> PMID: 29543905
88. Li W, Zhang C, Guo X, Liu Q, Wang K. Complete chloroplast genome of *Camellia japonica* genome structures, comparative and phylogenetic analysis. PLoS ONE. 2019; 14(5):e0216645. <https://doi.org/10.1371/journal.pone.0216645> PMID: 31071159
89. Li XW, Yang Y, Henry RJ, Rossetto M, Wang YT, Chen SL. Plant DNA barcoding: from gene to genome. Biol Rev. 2015; 90: 157–166. <https://doi.org/10.1111/brv.12104> PMID: 24666563
90. Jansen RK, Cai Z, Raubeson LA, Daniell H, Depamphilis CW, Leebens-Mack J, et al. Analysis of 81 genes from 64 plastid genomes resolves relationships in angiosperms and identifies genome-scale evolutionary patterns. P Natl Acad Sci USA. 2007; 104(49):19369–19374. <https://doi.org/10.1073/pnas.0709121104> PMID: 18048330
91. Williams AV, Miller JT, Small I, Nevill PG, Boykin LM. Integration of complete chloroplast genome sequences with small amplicon datasets improves phylogenetic resolution in *Acacia*. Mol Phylogenet Evol. 2016; 96:1–8. <https://doi.org/10.1016/j.ympev.2015.11.021> PMID: 26702955
92. Hernandez-Leon S, Gernandt DS, Perez de la Rosa JA, Jardon-Barbolla L. Phylogenetic relationships and species delimitation in *Pinus* section Trifoliae inferred from plastid DNA. PLoS One. 2013; 8(7):1–14. <https://doi.org/10.1371/journal.pone.0070501> PMID: 23936218
93. Lee YO, Kanno A, Kameya T. The physical map of the chloroplast DNA from *Asparagus officinalis* L. Theor Appl Genet. 1996; 92:10–14. <https://doi.org/10.1007/BF00222945> PMID: 24166110
94. Kanno A, Lee YO, Kameya T. The structure of the chloroplast genome in members of the genus *Asparagus*. Theor Appl Genet. 1997; 95:1196–1202.
95. Sheng W, Chai X, Rao Y, Tu X, Du S. Complete chloroplast genome sequence of *Asparagus* (*Asparagus officinalis* L.) and its phylogenetic position within Asparagales. J Plant Breed Genet. 2017; 5(3):121–128.
96. Li JR, Li SF, Wang J, Dong R, Zhu HW, Li N, et al. Characterization of the complete chloroplast genome of *Asparagus setaceus*. Mitochondrial DNA B Resour, 2019; 4(2):2639–2640. <https://doi.org/10.1080/23802359.2019.1643798> PMID: 33365661

97. Kurtz S, Phillippy A, Delcher AL, Smoot M, Shumway M, Antonescu C, et al. Versatile and open software for comparing large genomes. *Genome Biol.* 2004; 5(2):R12.1–R12.9. <https://doi.org/10.1186/gb-2004-5-2-r12> PMID: 14759262
98. Tillich M, Lehwark P, Pellizzer T, Ulbricht-Jones ES, Fischer A, Bock R, et al. GeSeq—versatile and accurate annotation of organelle genomes. *Nucleic Acids Res.* 2017; 45(W6):W11. <https://doi.org/10.1093/nar/gkx391> PMID: 28486635
99. Greiner S, Lehwark P, Bock R. OrganellarGenomeDRAW (OGDRAW) version 1.3.1: expanded toolkit for the graphical visualization of organellar genomes. *Nucleic Acids Res.* 2019; 47(W1):W59–W64. <https://doi.org/10.1093/nar/gkz238> PMID: 30949694
100. Beier S, Thiel T, Münch T, Scholz U, Mascher M. MISA-web: a web server for microsatellite prediction. *Bioinformatics.* 2017; 33:2583–2585. <https://doi.org/10.1093/bioinformatics/btx198> PMID: 28398459
101. Kurtz S, Choudhuri JV, Ohlebusch E, Schleiermacher C, Stoye J, Giegerich R. REPuter: The Manifold Applications of Repeat Analysis on a Genomic Scale. *Nucleic Acids Res.* 2001; 29(22):4633–4642. <https://doi.org/10.1093/nar/29.22.4633> PMID: 11713313
102. Frazer KA, Pachter L, Poliakov A, Rubin EM, Dubchak I. VISTA: computational tools for comparative genomics. *Nucleic Acids Res.* 2004; 32(Web Server issue):W273–W279. <https://doi.org/10.1093/nar/gkh458> PMID: 15215394
103. Brudno M, Malde S, Poliakov A, Do CB, Couronne O, Dubchak I, et al. Glocal Alignment: Finding Rearrangements During Alignment. *Bioinformatics.* 2003; 19(S1):i54–i62. <https://doi.org/10.1093/bioinformatics/btg1005> PMID: 12855437
104. Amiryousefi A, Hyvönen J, Pocza P. IRscope: an online program to visualize the junction sites of chloroplast genomes. *Bioinformatics.* 2018; 34(17):3030–3031. <https://doi.org/10.1093/bioinformatics/bty220> PMID: 29659705
105. Kato K, Rozewicki J, Yamada KD. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief Bioinform.* 2019; 20(4):1160–1166. <https://doi.org/10.1093/bib/bbx108> PMID: 28968734
106. Rozas J, Ferrer-Mata A, Sánchez-DeBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins S, et al. DNA Sequence Polymorphism (DnaSP). Version: 6.12.03 [software]. Universitat de Barcelona. 2019 Feb 26 [cited 10 Sep 2019]. Available from: <http://www.ub.edu/dnasp/>.
107. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol Biol Evol.* 2018; 35(6):1547–1549. <https://doi.org/10.1093/molbev/msy096> PMID: 29722887
108. Royal Horticultural Society Victoria. *Asparagus aethiopicus* L.; 2018 May 27 [cited 2021 Sep 14]. Horticultural Flora of South-eastern Australia (HORTFLORA) [Internet]. Available from: <https://hortflora.rbg.vic.gov.au/taxon/adab3b44-5340-11e7-b82b-005056b0018f>.
109. Moisan-thiery M, Marhadour S, Kerlan MC, Dessenne N, Perramant M, Gokelaere T, et al. Potato cultivar identification using simple sequence repeats markers (SSR). *Potato Res.* 2005; 48:191–200. <https://doi.org/10.1007/BF02742376>
110. Schneider K, Douches DS. Assessment of PCR-based simple sequence repeats to fingerprint North American potato cultivars. *Am Potato J.* 1997; 74(3):149–160.
111. Pereira-Lorenzo S, Ramos-Cabrer AM, Gonzalez-Diaz AJ, Diaz-Hernandez MB. Genetic assessment of local apple cultivars from La Palma, Spain, using simple sequence repeats (SSRs). *Sci Hortic.* 2008; 117(2):160–166. <https://doi.org/10.1016/j.scienta.2008.03.033>
112. Yu JK, Mangor J, Thompson L, Edwards KJ, Slabaugh MB, Knapp SJ. Allelic diversity of simple sequence repeats among elite inbred lines of cultivated sunflower. *Genome.* 2002; 45(4): 652–660. <https://doi.org/10.1139/g02-025> PMID: 12175068
113. Chen XQ, Kamilla GT. *Asparagus racemosus* Willdenow. In: Flora of China. Volume 24. Beijing: Science Press & St. Louis: Missouri Botanical Garden; 2000. pp. 210. Available from: <http://www.iplant.cn/foc/pdf/Liliaceae.pdf>.
114. Lee YO, Kanno A, Kameya T. Phylogenetic relationships in the genus *Asparagus* based on the restriction enzyme analysis of the chloroplast DNA. *Japanese Journal of Breeding.* 1997; 47(4):375–378. <https://doi.org/10.1270/jsbbs1951.47.375>