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Complete plastomes serve as desirable molecular makers for precise identification of *Asparagus cochinchinensis* (Asparagaceae) and nine other congeneric species frequently utilized as its adulterants

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Abstract

Backgroud The processed tuberous roots of *Asparagus cochinchinensis* (Asparagaceae), known as Asparagi Radix, have long been used in East Asia (particularly in China) as traditional medicines and play an indispensable role in the pharmaceutical industry. However, the frequent adulteration of Asparagi Radix with processed tuberous roots obtained from nine other congeneric species could potentially compromise the quality control measures for related pharmaceutical products, while also posing challenges to the conservation and rational exploitation of the nine adulterant congeneric species that are also used as traditional ethnomedicines. Given this issue, this study aims to develop a molecular authentication method for the accurate identification of *A. cochinchinensis* and the nine congeneric adulterants, employing the genome skimming approach to generate complete plastid genomes (plastomes) and nuclear ribosomal DNA (nrDNA) arrays as the candidate molecular markers.

Results Through comprehensive phylogenetic and genetic distance analyses based on extensive sampling at both inter- and intra-specific levels, the efficacy of the two candidate molecular markers was assessed by investigating whether their inter-specific genetic divergences align with the taxonomically delineated species boundaries.

Conclusion The results indicated that complete plastomes exhibit superior performance for accurately identifying *A. cochinchinensis* (the botanical source of Asparagi Radix) and the nine congeneric adulterants, thus can serve as

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the optimal molecular markers for effective authentication of Asparagi Radix. The desirable discriminative power demonstrated by complete plastomes suggests that the PCR-free molecular authentication method developed in this study will not only contribute to the quality control of pharmaceutical products derived from Asparagi Radix but also facilitate the conservation efforts and rational exploitation of the nine *Asparagus* species commonly used as adulterants.

Keywords Adulterants, Complete plastomes, DNA barcoding, Medicinal plants, Species identification

Background

The monocotyledonous genus *Asparagus* L., which encompasses approximately 215 species distributed widely across temperate and tropical regions in Africa and Eurasia, represents the largest genus within the Asparagaceae subfamily Asparagoideae [1–4]. *Asparagus* exemplifies a renowned genus within the order Asparagales, characterized not only by its diverse life forms comprising perennial herbs, subshrubs, and vines, but also by its substantial economic importances spanning medicinal, ornamental, and culinary domains [5–9]. There are a total of 31 *Asparagus* species documented in China, comprising 29 indigenous and two introduced species [10]. Among these *Asparagus* species documented in China, a total of ten are commonly utilized for their therapeutical properties. The well-known traditional Chinese medicine, Asparagi Radix, is the processed tuberous roots of *Asparagus cochinchinensis* [11]. The therapeutic properties of Asparagi Radix have been extensively utilized for over two millennia in East Asia, encompassing China, Japan, Korea, and Vietnam, for the treatment of a vast array of diseases including gastric pain, constipation, pyrexia, bronchial asthma, coughing fits, rhinitis and tracheitis symptoms, as well as pneumonia and thrombosis conditions. Moreover, it has also shown therapeutical efficacy in managing diabetes mellitus complications such as cataracts while addressing dermatological issues like acne vulgaris and urticaria [12–15]. The comprehensive therapeutic properties of Asparagi Radix have served as the foundation for the development of over 180 pharmaceuticals and health products, utilizing it as a key raw material (https://db.yaozh.com/zhongyaocai). Additionally, the other nine medicinal *Asparagus* species distributed in China, namely *A. dauricus*, *A. filicinus*, *A. meioclados*, *A. lycopodineus*, *A. myriacanthus*, *A. officinalis*, *A. oligoclonos*, *A. schoberioides*, and *A. taliensis*, are also widely utilized as traditional medicinal herbs by diverse ethnic communities in China [10, 16, 17], despite their exclusion from the Chinese Pharmacopoeia.

Recently, there has been a growing demand for raw materials from pharmaceutical enterprise, which has facilitated the commercial cultivation of *A. cochinchinensis* with a specifical focus on yielding sufficient Asparagi Radix for pharmaceutical manufacturing purposes; nevertheless, due to the inadequate cultivation scale, extensive harvesting of the tuberous roots from wild *A. cochinchinensis* populations still persists [18–20]. Notably, *A. cochinchinensis* exhibits a wide distribution range across China, Japan, Korea, and Vietnam [13], while also displaying high degrees of morphological resemblances to the aforementioned nine medicinal *Asparagus* species [10]. The scarcity of taxonomic expertise among professionals engaged in the collection of medicinal plants and the manufacturing of relevant plant products consequently leads the extensive harvesting of the tuberous roots of the other nine congeneric species from their wild populations, followed by their processing into commercial products marketed as Asparagi Radix for pharmaceutical purposes [17]. Considering the significant variations in chemical compositions and pharmacological properties between *A. cochinchinensis* and the nine congeneric species, this problematic practice could potentially pose adverse effects on the quality and efficacy of commercial pharmaceuticals that utilize Asparagi Radix as their raw materials [9, 14, 15, 21]. Additionally, among these nine *Asparagus* species traditionally utilized as ethnic medicines but mistakenly employed as the adulterants of Asparagi Radix, *A. meioclados*, *A. myriacanthus*, and *A. tailiensis* exhibit relatively small natural population sizes and restricted distribution ranges [10]. Previous studies have demonstrated that the overexploitation of plant species with limited distribution and small population sizes likely renders them more susceptible to extirpation from natural populations [22, 23]. Therefore, the extensive harvesting of tuberous roots from natural habitats for pharmaceutical purposes would further intensify their vulnerability, potentially leading them towards endangerment due to the partial exhaustion or extinction of their natural populations. Furthermore, the depletion of wild resources will not only hamper the therapeutical utilization of these medicinal *Asparagus* species by local ethnic communities but also impede scientific investigations aimed at exploiting bioactive specified metabolites with potential pharmacological properties within them. Overall, the indiscriminate harvesting of these congeneric medicinal species from their wild populations for commercial purposes would lead to a series of detrimental consequences. Therefore, it is crucial to accurately identify *A. cochinchinensis* and the nine commonly harvested congeneric species to ensure the efficacy of commercial pharmaceuticals that utilize Asparagi Radix as a raw material. Additionally, this measure will also promote

conservation efforts and rational exploration of the wild resources of the nine medicinal *Asparagus* species, which are frequently used as adulterants for *A. cochinchinensis*.

Due to the substantial morphological resemblance observed between *A. cochinchinensis* and the nine medicinal congeneric species mistakenly employed as the adulterants of Asparagi Radix, the accurate identification of these congeners based on morphology represents a complex challenge in the absence of taxonomical experts. The emergence of DNA barcoding, a technique that standardizes the utilization of single- or multiple-locus DNA sequence regions for species identification [24], presents a potential resolution to this dilemma. Over the past few decades, three plastid loci *mat*K, *rbc*L, and *psb*A-*trn*H, along with the nuclear internal transcribed spacer (ITS), have been extensively employed as standard DNA barcodes for plant species identification using polymerase chain reaction (PCR) and Sanger sequencing technologies, and the application of standard DNA barcodes has demonstrated robust performance across diverse plant lineages [24–27]. However, previous studies have manifested that standard DNA barcodes (such as *trn*H-*psb*A, *mat*K, and ITS) display limited sequence variations among *Asparagus* species, indicating their weak discriminative power for accurate species identification within this economically important plant lineage [17, 28, 29].

Recently, the rapid advancement of the high throughput sequencing technologies has greatly facilitated effective compensation for the limitations of DNA barcoding technique in accurately identifying plant species. For instance, the genome skimming approach, which employs high throughput sequencing technologies to generate low-coverage genome sequencing data for the assemblance of complete plastid genomes (plastomes) and nuclear tandemly repeated ribosomal DNA (nrDNA) arrays as molecular markers for plant phylogenetic analyses and species identification [30], has been recommended as an innovative method to further optimize plant DNA barcoding technique [26]. The complete plastomes and nrDNA arrays, in comparison to standard DNA barcodes, possess a significantly greater sequence length and exhibit higher number of sequence variations among closely related species; the application of both datasets in plant species identification, therefore, demonstrates substantial potential for resolving the intricacies that remain unsolved by the application of standard DNA barcodes [30–36]. Furthermore, previous studies have demonstrated that the application of genome skimming approach enables the convenient generation of complete plastomes and nrDNA arrays, even from plant materials containing minute quantities of highly degraded genomic DNA, such as long term preserved herbarium specimens and processed plant products [37–40], thereby showcasing the practical feasibility of utilizing plastomes and nrDNA arrays as the molecular makers to identify the source species of the processed tuberous roots marketed as Asparagi Radix.

Given the afore-mentioned advantages of employing genome skimming approach for plant species identification, this study aims to develop a PCR-free method that enables effective authentication of Asparagi Radix based on the comprehensive assessment of the discriminatory power of complete plastomes and nrDNA arrays for accurately identifying *A. cochinchinensis* (the botanical source of Asparagi Radix) and the nine commonly collected adulterant congeneric species. To accomplish this objective, the following two inquiries were specifically addressed: (1) Does the utilization of complete plastomes and nrDNA arrays greatly enhance the efficacy of species identification in comparison to standard DNA barcodes? (2) Regarding complete plastomes and nrDNA arrays, which can serve as the optimal molecular marker for accurate identification of the ten target *Asparagus* species?

Methods

Plant material sampling and high throughput genome sequencing

A total of 42 individual plants, representing 21 Asparagus species (Table S1), were collected from their natural habitats. Leaf tissues were gathered and desiccated using silica gel to facilitate the extraction of genomic DNA. For the ten target species, i.e., *A. cochinchinensis*, *A. dauricus*, *A. filicinus*, *A. meioclados*, *A. lycopodineus*, *A. myriacanthus*, *A. officinalis*, *A. oligoclonos*, *A. schoberioides*, and *A. taliensis*, multiple intra-specific accessions from diverse distribution localities were sampled. Identification of these Voucher specimens (Table S1) were verified by Dr. Yunheng Ji and deposited in the Herbarium of Kunming Institute of Botany, the Chinese Academy of Sciences (KUN).

The total genomic DNA of each sample was extracted from the silica gel-dried leaf tissues using the modified CTAB method [41]. Paired-end shotgun libraries were prepared utilizing a TruSeq DNA Sample Prep Kit (Illumina, Inc., San Diego, CA, USA) with an average insert size of 400 bp in accordance with the manufacturer's protocol. Subsequently, low coverage genome sequencing was conducted on the high throughput genome sequencing platform Illumina NovaSeq 6000 instrument (Illumina, Inc., San Diego, CA, USA) to generate approximately two Gbp of paired-end raw reads (2 × 150 bp) for each sample.

Assembly and annotation of complete plastomes and NrDNA arrays

The raw Illumina reads were filtered using fastP v0.15.0 [42] with parameters '-n 10' and '-q 15' to remove

adaptors and low-quality reads. Subsequently, the complete plastome and nr DNA arrays of each sample were assembled based on the clean Illumina reads using the GetOrganelle ver. 1.6.3a pipeline [43] with default parameters and preset options, employing *A. cochinchinensis* plastome (MZ424304) and *Heteropolygonatum alternicirrhosum* nr DNA arrays (MZ493128) as the references. The assembled plastomes and nr DNA arrays were aligned to the reference sequences using MAFFT v7.450 [44] for evaluating the assembly completeness with Geneious v10.2.3 [45]. All assembled plastomes were annotated using the Geseq v2.03 [46] with default parameters. Circular plastome maps were generated using the online tool OrganellarGenomeDRAW [47]. The annotation of assembled nrDNA arrays was conducted by comparing them with the nrDNA of the *H. alternicirrhosum* reference sequence (MZ493128) to identify sequence regions corresponding to 18 S rRNA, ITS1, 5.8 S rRNA, ITS2, and 28 S rRNA using Geneious v10.2.3 [45].

Data analysis

In addition to the plastomes and nrDNA arrays generated in this study, seven complete plastomes of *Asparagus* species were retrieved from the NCBI GenBank database (Table S2) for investigating their discriminative power in distinguishing the ten target *Asparagus* species. In total, 49 complete plastomes representing 22 *Asparagus* species and 42 nrDNA arrays from 21 *Asparagus* species were utilized to assess their effectiveness in discriminate among the ten target *Asparagus* species. Additionally, standard DNA barcodes including plastid *rbc*L, *mat*K, and *trn*H-*psb*A sequences extracted from each complete plastome as well as nuclear ITS region obtained from nrDNA arrays were compared with complete plastomes and nrDNA arrays to evaluate their discriminatory power in identifying the ten target *Asparagus* species.

The discriminative power of completed plastomes and nrDNA arrays, along with the four standard DNA barcodes in terms of identifying the ten target *Asparagus* species was investigated through phylogenetic and genetic distance analyses. For each dataset, DNA sequences were aligned using MAFFT v7.450 [44]. Sequence variations of each dataset were then investigated using MEGA X [48] to identify sequence variations and corresponding parsimony informative sites. The Maximum Likelihood (ML) method was employed to conduct phylogenetic analysis for each dataset. The ML phylogenies were reconstructed using the online software IQ-TREE 2v2.1.3 [49], and 1,000 rapid bootstrap (BS) replicates were performed to estimate the support value for each node. To establish rooted tree topologies, three closely related taxa, namely *Liriope muscari*, *Ophiopogon japonicus*, and *H. alternicirrhosum*, which are closely related to the genus *Asparagus* [50], were selected as the outgroup for phylogenetic analysis. The discriminative power of each dataset in identifying the ten target *Asparagus* species was evaluated by examining the species-level monophyly of these taxa, as inferred from ML tree topologies.

The discriminative power of each dataset for identifying the ten target *Asparagus* species was also evaluated through the presence or absence of barcoding gaps, where the minimum interspecific genetic variation surpasses the maximum intraspecific genetic variation [51]. The magnitudes of both interspecific and intraspecific genetic variations were assessed by calculating pairwise genetic distances for each dataset separately using the Kimura 2-parameter (K2P) model in MEGA X [52]. The genetic distance analyses were performed based on the respective alignment of complete plastomes, nrDNA arrays, plastid *rbc*L, *mat*K, and *trn*H-*psb*A sequences, as well as nuclear ITS regions. Scatter plots representing genetic variations were created to visually present the minimum interspecific distances in contrast to the maximum intraspecific distances across the six datasets, thereby illustrating the presence or absence of barcoding gaps at the species level.

Results

The assembly and annotation of complete plastomes and NrDNA arrays

A range of 977,972 to 38,832,514 paired-end clean reads were generated by Illumina sequencing for the 42 samples. Based on the low-coverage genome sequencing data, the complete plastome and entire nrDNA arrays for each sample were successfully assembled. The summary of Illumina sequencing and assembly of complete plastome and nrDNA arrays for each sample is presented in Table S3. The genome sizes of these newly assembled plastomes ranged from 156,208 bp to 157,139 bp (with sequencing coverage varying from 312.82 to 2,405.366 times), which consistently possessed the characteristic quadripartite structure comprising a large single copy (LSC) region and a small single copy (SSC) region separated by two inverted repeat (IR) copies (Fig. S1). Plastome annotation identified a total of 114 unique plastid genes within each plastome, including 80 protein-coding genes, 30 tRNAs, and four rRNAs (Table S4). Additionally, the assembly of nrDNA arrays provided full coverage of the 18 S rDNA, ITS1, 5.8 S rDNA, ITS2, and 28 S rDNA regions in each sample, with the sequencing coverage varying from 702.471 to 5,385.597 times (Table S3).

Phylogenetic inferences

The alignment of complete plastomes yielded a matrix of 163,892 bp, encompassing 9,788 variable sites and 7,908 parsimony informative sites (Table S5). The matrix of nrDNA arrays was 5,870 bp in length and consisted of

488 variable sites along with 337 parsimony informative sites (Table S5). In comparison to the three plastid loci (*rbc*L, *mat*K, *trn*H-*psb*A) and the nuclear ITS sequence regions subsampled from the two datasets, both the complete plastome and nrDNA datasets exhibited significantly higher levels of sequence variations that provided more comprehensive phylogenetic information. Specifically, the *rbc*L matrix (1,443 bp in length) contained 90 sequence variations and 60 parsimony informative sites; the *mat*K matrix (1,563 bp in length) displayed 125 sequence variations and 86 parsimony informative sites; the *trn*H-*psb*A matrix (557 bp in length) included 47 sequence variations and 29 parsimony informative sites; while the ITS matrix (686 bp in length) possessed 270 sequence variations and 197 parsimony informative sites (Table S5).

The ML phylogenetic analysis of complete plastomes (Fig. 1; Table 1) successfully resolved all the ten target species as monophyletic entities with robust bootstrap values, while the ML phylogeny based on nrDNA arrays failed to resolve the species-level monophyly of *A. taliensis* (Fig. 2; Table 1). Comparatively, the ML phylogenetic

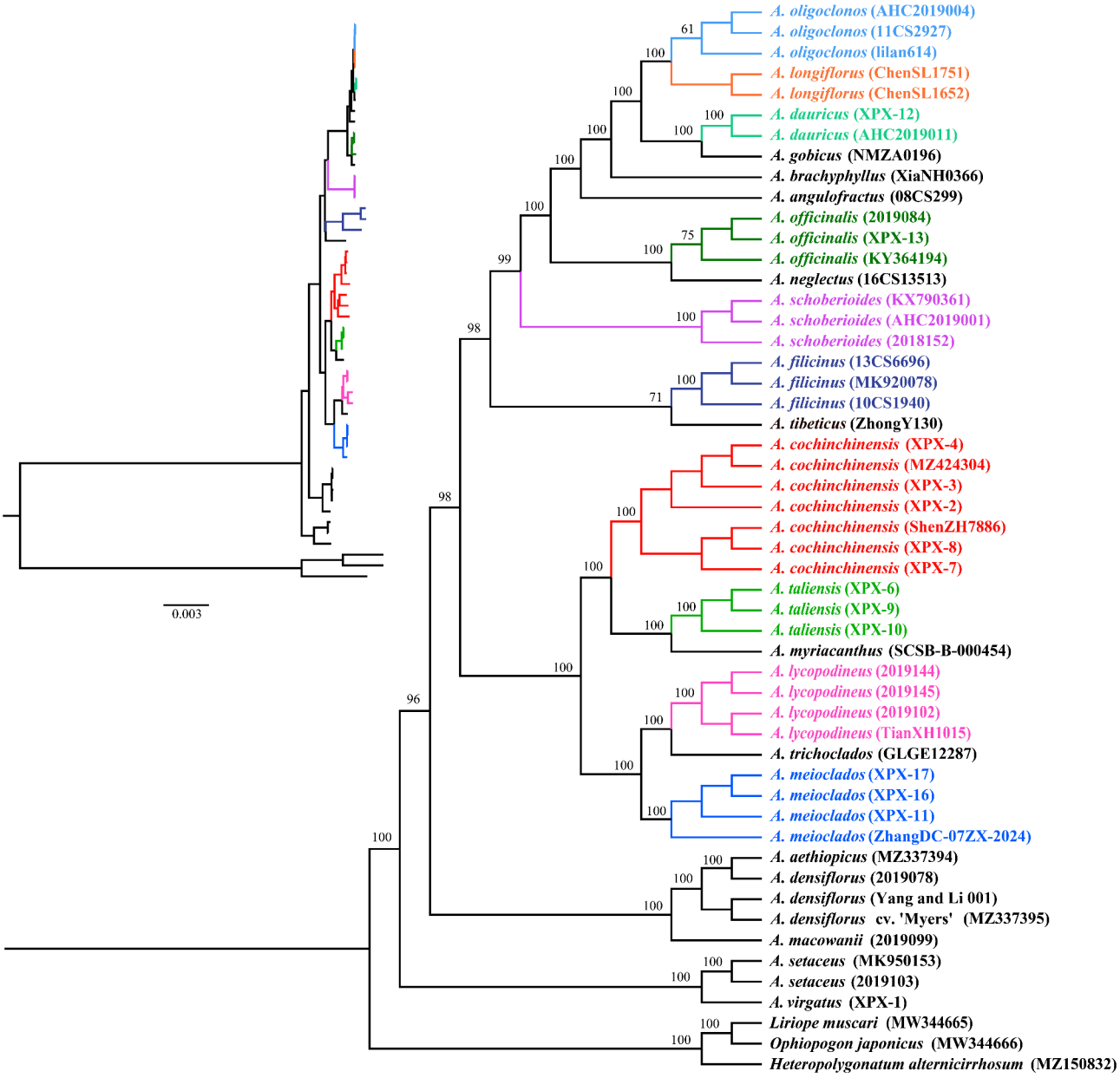

Fig. 1 Cladogram of the maximum likelihood (ML) phylogeny inferred from complete plastome sequences. Numbers at nodes represent the ML bootstrap values (BS) percentage. Tree topology with branch length is shown on the top left portion of the figure, and the scale bar below represents the branch length scale

Table 1 Species-level monophyly of the ten Chinese medicinal *Asparagus* species with multiple individuals sampled and branch support recovered by ML phylogenetic analysis of complete plastomes, Nr DNA, and four standard DNA barcodes (ITS, *rbc*L, *mat*K, and *trn*H-*psb*A)

Species	Plastome		nr DNA		ITS		rbcL		matK		trnH-psbA	
	Monophyly	BS (%)	Monophyly	BS (%)	Monophyly	BS (%)	Monophyly	BS (%)	Monophyly	BS (%)	Monophyly	BS (%)
A. cochinchinensis	+	100	+	65	+	66	+	39	-	-	-	-
A. lycopodineus	+	100	+	99	+	87	-	-	+	29	-	-
A. meioclados	+	100	+	85	+	67	+	35	+	58	-	-
A. filicinus	+	100	+	100	+	100	+	97	-	-	+	73
A. officinalis	+	75	+	89	+	55	+	63	+	39	+	28
A. taliensis	+	100	-	-	-	-	-	-	+	72	-	-
A. dauricus	+	100	+	85	-	-	-	-	+	65	-	-
A. longiflorus	+	99	+	98	+	99	-	-	-	-	-	-
A. oligoclonos	+	61	+	66	+	64	-	-	-	-	-	-
A. schoberioides	+	100	+	100	+	100	+	99	+	99	-	-

Abbreviations: ML, maximum-likelihood; BS, bootstrap; Positive sign (+), recovered; Negative sign (-), unrecovered

analysis of ITS sequences failed to resolve *A. taliensis* and *A. dauricus* as monophyletic entities (Fig. 3; Table 1). Additionally, the ratio of monophyletic resolution for the ten target *Asparagus* species by phylogenetic analysis of *mat*K (6 out of 10) (Fig. 4; Table 1), *rbc*L (5 out of 10) (Fig. 5; Table 1), and *trn*H-*psb*A (2 out of 10) (Fig. 6; Table 1) datasets was relatively lower compared to that obtained from ITS phylogeny (8 out of 10).

Barcoding gap analysis

In accordance with the phylogenetic tree toplogies, distinct barcoding gaps were observed among the ten target *Asparagus* species through the genetic distance analysis of complete plastome dataset. This is evidenced by the non-overlap between interspecific and intraspecific K2P distances (Fig. S2). Comparatively, the genetic distance analysis of nr DNA arrays (Fig. S3), ITS (Fig. S4), *matK* (Fig. S5), *rbcL* (Fig. S6), and *psbA-trnH* (Fig. S7) datasets revealed varying degrees of overlap between interspecific and intraspecific K2P distances among the ten target species, suggesting an absence of clear barcoding gap within these five datasets.

Discussion

Due to the morphological complexity observed among closely related *Asparagus* species and the limited discriminative power of standard DNA barcodes [17, 28, 29], the accurate identification of *A. cochinchinensis* (the botanical source of Asparagi Radix) and the nine congeneric species (i.e., *A. dauricus*, *A. filicinus*, *A. meioclados*, *A. lycopodineus*, *A. myriacanthus*, *A. officinalis*, *A. oligoclonos*, *A. schoberioides*, and *A. taliensis*) whose tuberous roots are frequently collected as the adulterants of Asparagi Radix pose substantial challenges. Given the considerably higher levels of sequence variations observed in complete plastomes and nrDNA arrays compared to the four standard DNA barcodes (i.e., plastid *rbc*L, *mat*K, *trn*H-*psb*A, and nuclear ITS sequence regions) subsampled from the two datasets, their utilization as molecular markers for plant species identification offers a substantial enhancement in species identification resolution across diverse angiosperm taxa [36, 53–61]. Since the effective authentication of Asparagi Radix will not only contribute to the quality control of its pharmaceutical products but also promote conservation efforts and rational exploration of the wild resources of those medicinal *Asparagus* species that are commonly used as adulterants for *A. cochinchinensis*, this study aims to develop a PCR-free molecular authentication method that employes the genome skimming approach to generate complete plastomes and nrDNA arrays as molecular markers for the accurate identification of the ten target *Asparagus* species.

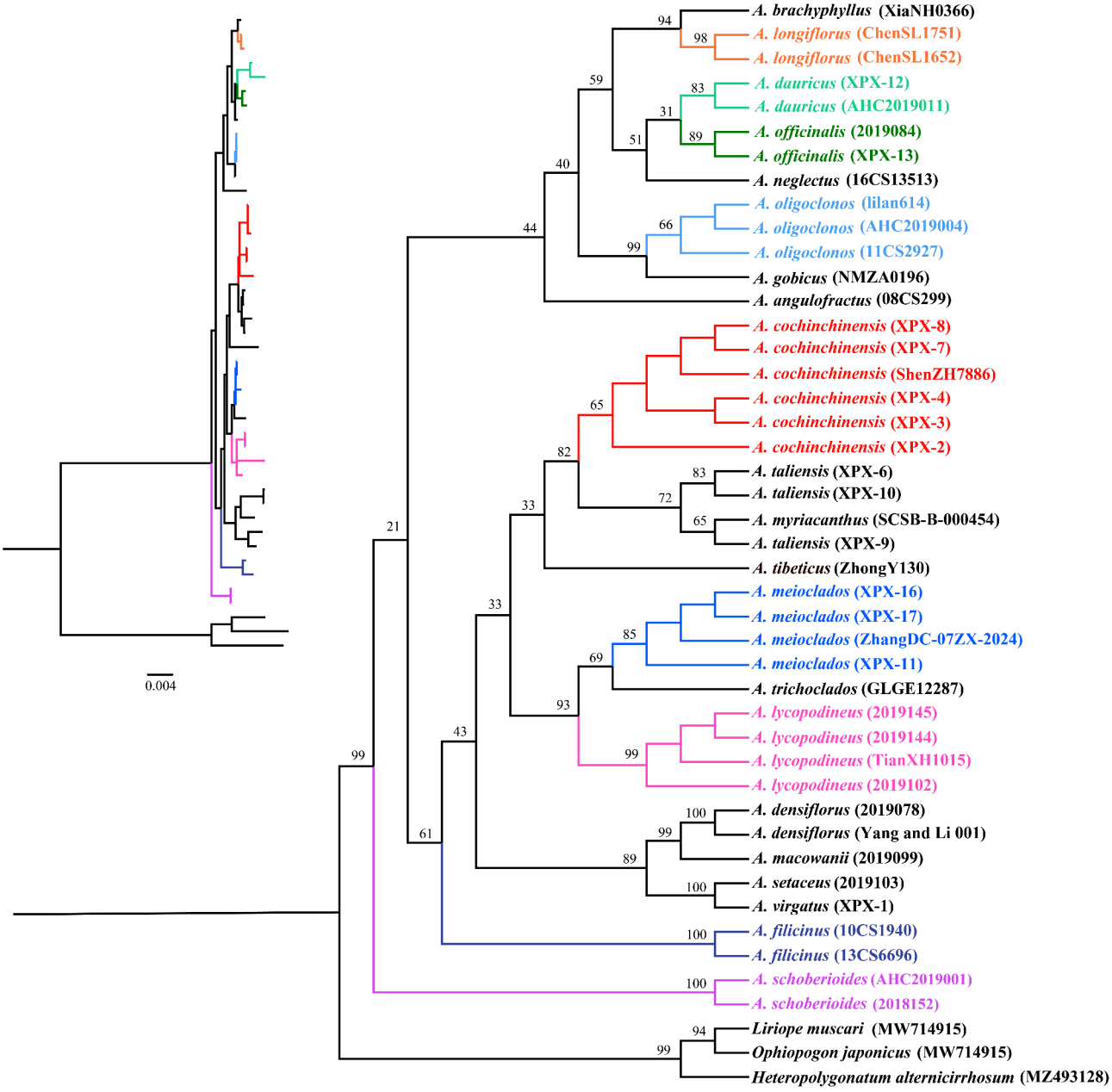

Fig. 2 Cladogram of the maximum likelihood (ML) phylogeny inferred from nuclear ribosomal DNA (nrDNA) arrays. Numbers at nodes represent the ML bootstrap values (BS) percentage. Tree topology with branch length is shown on the top left portion of the figure, and the scale bar below represents the branch length scale

Notably, previous studies have also indicated that both complete plastomes and nrDNA arrays demonstrate insufficient performance for species identification, particularly within plant lineages that have extensively undergone either recent radiative diversification or reticulate evolution events [21, 22, 62–64]. Therefore, conducting a comprehensive investigation into the discriminatory power of complete plastomes and nrDNA arrays will ensure the reliability of the molecular authentication method developed in this study. By extensively sampling Chinese *Asparagus* species and multiple intraspecific samples of the ten target *Asparagus* species, we conducted a comprehensive assessment with respects to the discriminative efficacy of complete plastomes and nrDNA arrays as well as three plastid loci (*mat*K, *rbc*L, and *trn*H-*psb*A) and nuclear ITS regions subsampled from the two datasets to investigate whether the interspecific genetic divergences of these molecular makers coincide with the morphologically delineated species boundaries.

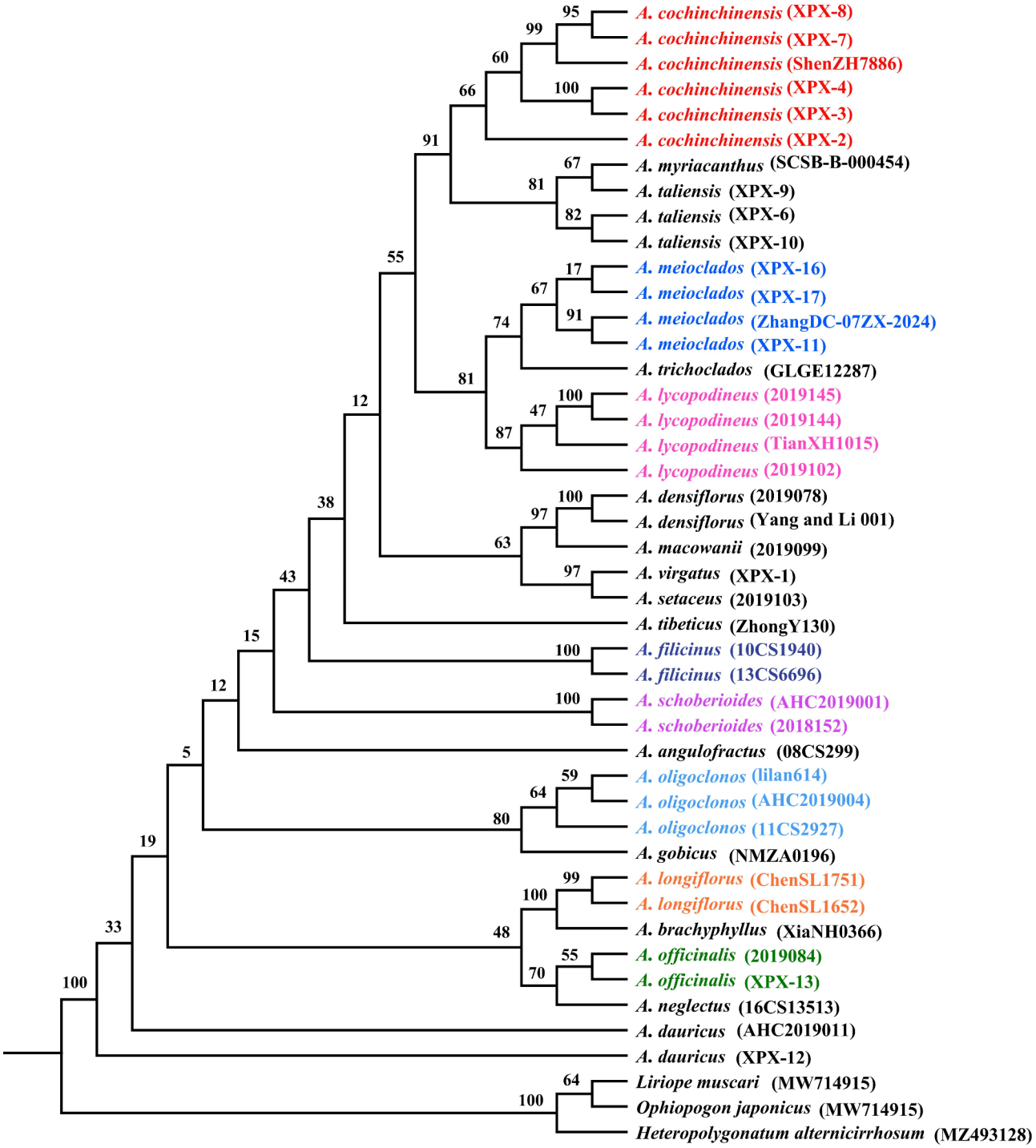

Fig. 3 Cladogram of the maximum likelihood (ML) phylogeny inferred from nuclear ITS sequence regions. Numbers at nodes represent the ML bootstrap values (BS) percentage

In accordance with theoretical predictions [26, 30, 31, 33, 37], our findings indicate that the utilization of complete plastomes and nrDNA arrays can enhance the accuracy of species identification compared to standard DNA barcodes. Furthermore, complete plastomes are more suitable than nrDNA arrays as optimal molecular markers for precise identification of *A. cochinchinensis* and other nine congeneric species commonly used as adulterants in Asparagi Radix. Specifically, the maximum likelihood (ML) phylogenetic analyses revealed that the discriminatory power of the four standard DNA barcodes (ITS, *mat*K, *rbc*L, and *trn*H-*psb*A) was relatively weaker

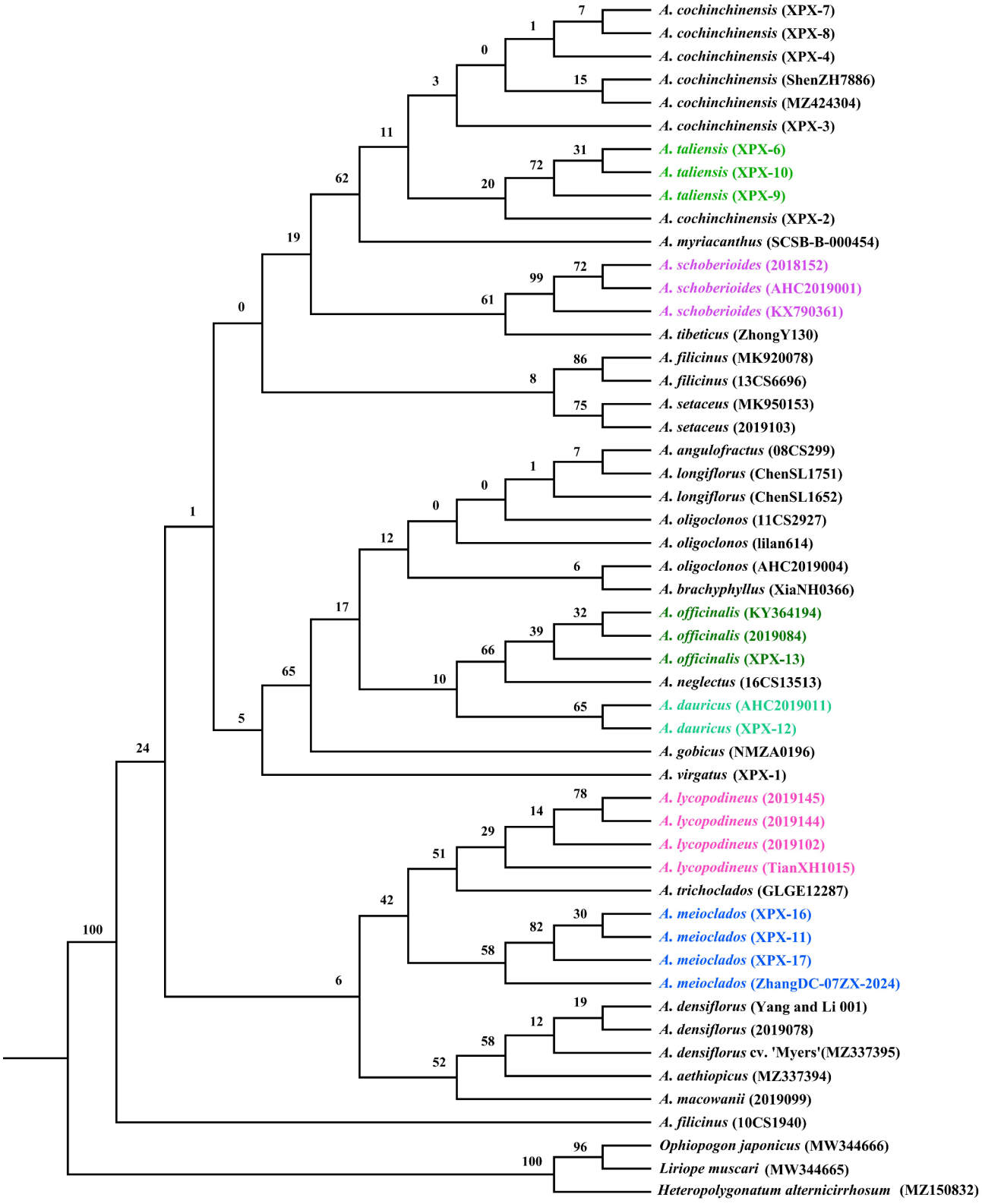

Fig. 4 Cladogram of the maximum likelihood (ML) phylogeny inferred from plastid *mat*K genes. Numbers at nodes represent the ML bootstrap values (BS) percentage

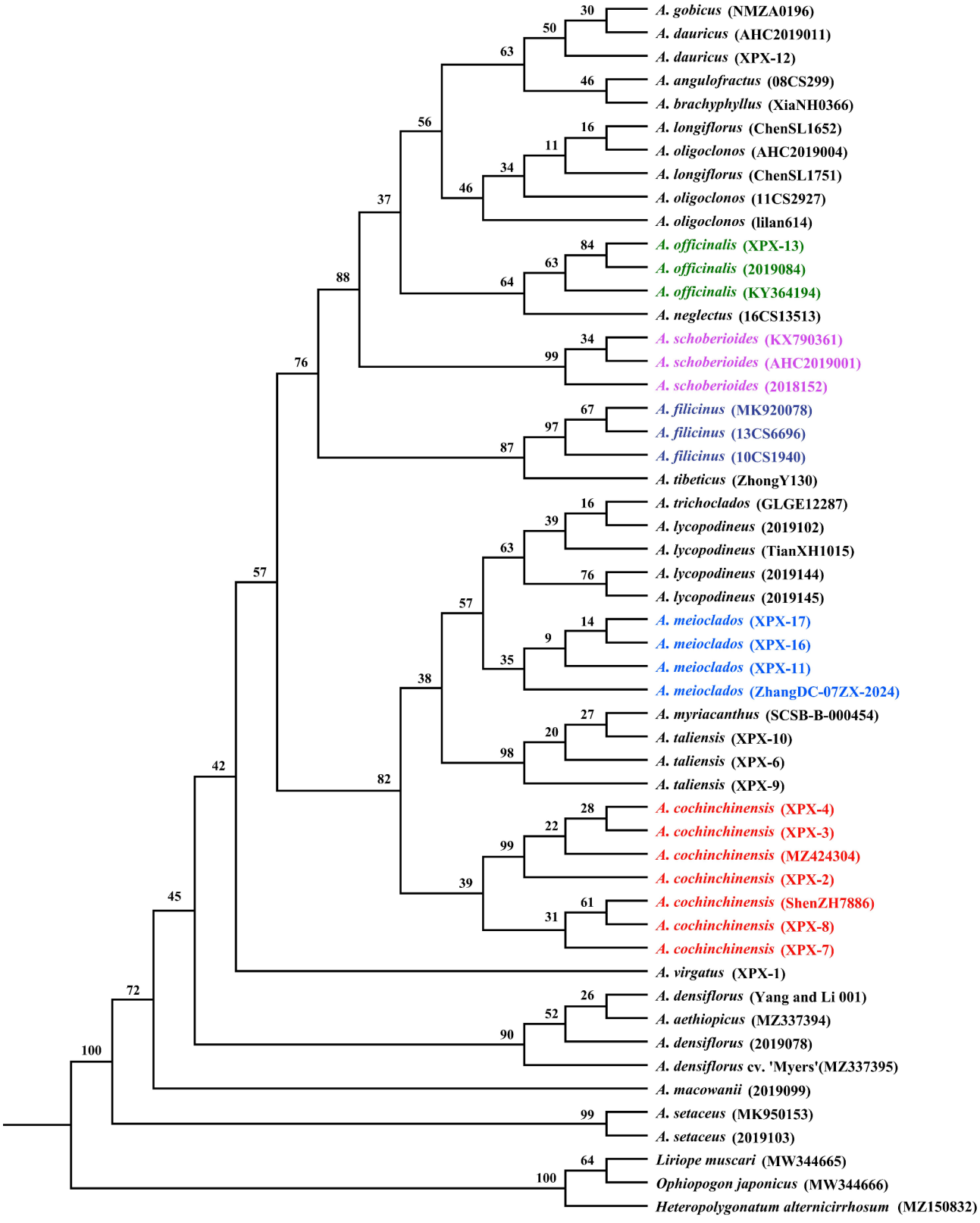

Fig. 5 Cladogram of the maximum likelihood (ML) phylogeny inferred from plastid *rbc*L genes. Numbers at nodes represent the ML bootstrap values (BS) percentage

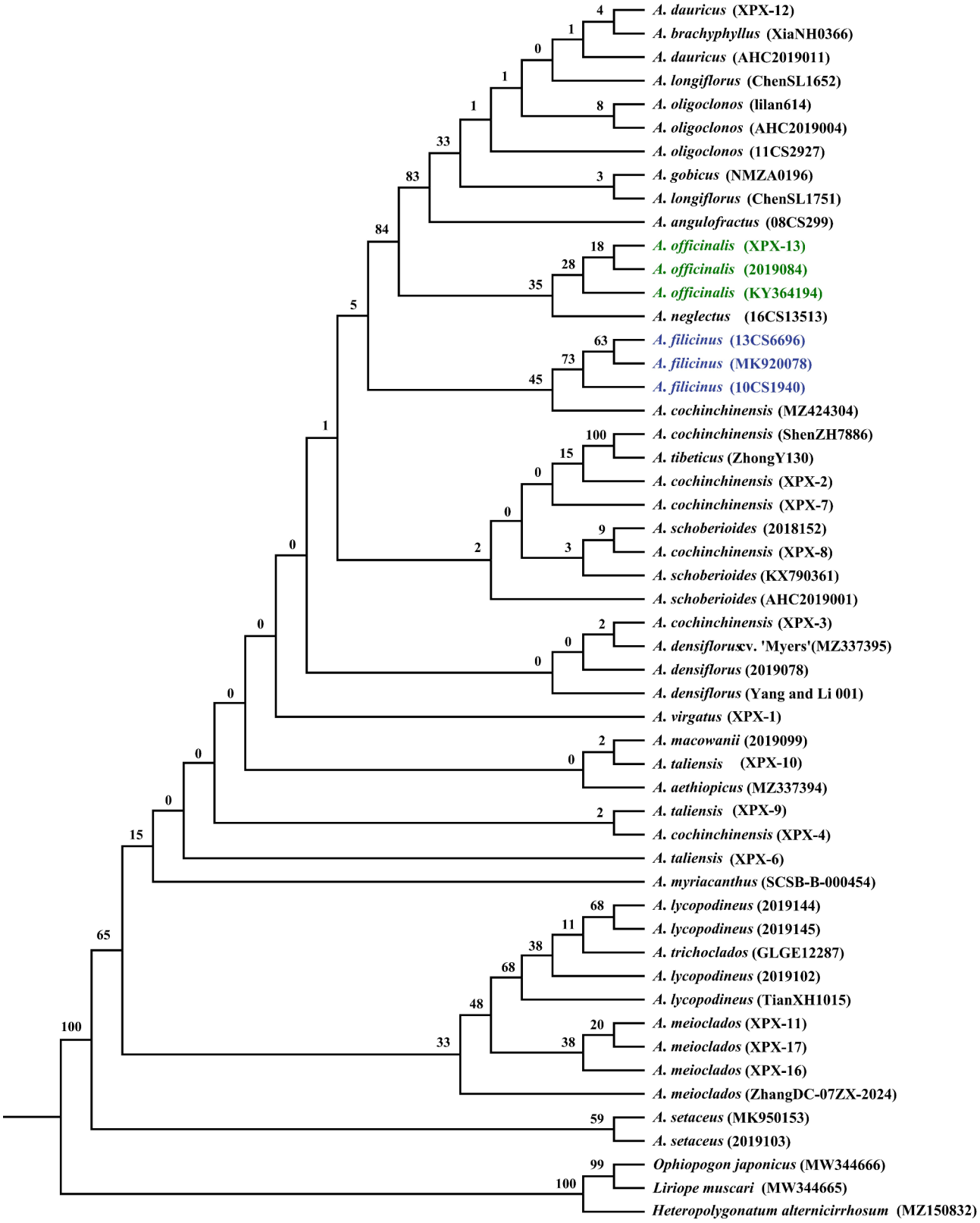

Fig. 6 Cladogram of the maximum likelihood (ML) phylogeny inferred from plastid *trn*H-*psb*A sequence regions. Numbers at nodes represent the ML bootstrap values (BS) percentage

in accurately identifying the ten target *Asparagus* species when compared to complete plastomes and nuclear ribosomal DNA arrays. The monophyletic resolution ratios for the ten target *Asparagus* species obtained from ML phylogenetic analyses using ITS, *mat*K, *rbc*L, and *trn*H-*psb*A datasets are comparatively lower than those achieved from complete plastome (100%) and nrDNA (nine out of ten) phylogenies. This finding suggests that both complete plastomes and nrDNA arrays exhibit a higher degree of consistency with species boundaries in terms of interspecific genetic divergence compared to standard DNA barcodes, thereby enhancing their efficacy in accurately identifying the ten target *Asparagus* species. Additionally, ML phylogenetic analysis based on complete plastomes successfully resolved all ten target *Asparagus* species as monophyletic units while failing to resolve the species-level monophyly of *A. taliensis* in ML phylogeny based on nrDNA arrays, highlighting the superior performance of complete plastomes in discriminating among these ten target *Asparagus* species. In accordance with this, genetic distance analysis of complete plastomes revealed the presence of distinct barcoding gaps among all ten target *Asparagus* species. In contrast, the analyses of nrDNA arrays, *rbcL*, *matK*, and *trnH-psbA* detected varying degrees of overlap between interspecific and intraspecific genetic distances within the ten target *Asparagus* species, indicating their absence of clear barcoding gaps. The findings indicate that the interspecific genetic differentiation of complete plastomes among the ten target *Asparagus* exhibits a high degree of congruence with morphologically delineated species boundaries. The results of phylogenetic and genetic distance analyses provide mutually supported evidence indicating that the utilization of complete plastomes as molecular markers enables precise identification of the ten targeted *Asparagus* species.

The analysis of sequence variations and corresponding parsimony informative sites across the six datasets revealed that the complete plastome dataset contains a substantial number of sequence variations and a high frequency of parsimony informative sites (Table S5), consistent with its superior discriminative power as demonstrated by phylogenetic and genetic distance analyses. Specifically, the maximum likelihood (ML) phylogenetic analysis of complete plastomes indicated that the majority of nodes within the tree topology were strongly supported (BS > 95%), whereas the ML phylogenies inferred from the other five datasets arrays exhibited relatively lower support (BS < 95%) for most nodes. This indicates a limited amount of phylogenetic information within the nrDNA, ITS, matK, rbcL, and psbA-trnH dataset [65], potentially resulting in an inability to resolve the species-level monophyly of certain *Asparagus* species as well as the absence of clear barcoding gaps. Moreover, ML phylogenies based on complete plastomes and nrDNA arrays exhibited a certain degree of incongruences in their respective tree topologies. The observed phylogenetic incongruence between plastid and nuclear genomic data implies potential hybridization or incomplete lineage sorting (ILS) events may have occurred during the evolutionary history of *Asparagus*, which could result in shared molecular markers among closely related species and thus do not align with species boundaries [26, 66]. Given the current lack of robust evidence, this hypothesis requires rigorous validation through the analysis of gene tree discordance and reticulate evolution, combined with incomplete ILS simulations, utilizing nuclear genome data encompassing a multitude of single-copy orthologous genes.

Based on comparative analysis of *Asparagus* plastomes, previous studies have identified several hypervariable sequence regions, including *accD-psaI*, *ccsA*, *trnS-trnG*, *ycf1*, and *rpl32-trnL* loci, which can serve as effective molecular markers for phylogenetic analysis and species identification [67, 68]. Although the application of these plastid loci for species identification offers advantages in terms of time and cost efficiency, the recovery of these DNA sequences from plant materials exclusively relies on Polymerase Chain Reaction (PCR) and Sanger sequencing. Given that genomic DNA of numerous commercial plant products has undergone extensive degradation during processing, the scarcity of PCR-amplifiable genomic DNA poses a formidable challenge in recovering target sequence regions from these processed plant materials [37, 38, 69]. This limitation consequently restricts the practical application of these plastid loci in the authentication of Asparagi Radix, where genomic DNA may have undergone substantial degradation degraded. Therefore, it is imperative to develop a PCR-free method for the authentication of Asparagi Radix.

The desirable discriminative power exhibited by complete plastomes suggests that the application of genome skimming approach to generate them as molecular markers can serve as an effective method for accurate identification of *A. cochinchinensis* and the other nine medicinal *Asparagus* species commonly employed as adulterants in Asparagi Radix. Notably, previous studies have demonstrated that recovering complete plastomes from processed products of medicinal plants or DNA-poor botanical materials (such as herbarium specimens) using high-throughput sequencing technologies is not an insurmountable challenge [37–40]. Therefore, the utilization of complete plastomes as molecular markers may offer an effective solution for authentication of commercial Asparagi Radix that lack PCR amplifiable genomic DNA. In view of this, the genome skimming approach demonstrates considerable potential for generating complete plastomes from processed tuberous roots of these

medicinal *Asparagus* species, serving as molecular markers to accurately determine if commercial Asparagi Radix is adulterated with any of the nine congeneric species.

The availability of taxonomically well-curated and high-resolution DNA barcode reference libraries is a fundamental prerequisite for the effective application of DNA barcoding techniques in plant species identification [27, 70, 71]. Given the remarkable discriminative power demonstrated by complete plastomes in identifying *A. cochinchinensis* and the other nine medicinal *Asparagus* species commonly used as adulterants in Asparagi Radix, the complete plastome dataset generated in this study can serve as the reference library for effective authentication of commercial Asparagi Radix. Owing to the advantages of recent advancements in high throughput sequencing technologies, retrieving complete plastomes from processed plant products containing minute quantities of highly degraded genomic DNA has become a more feasible task [38]. Therefore, accurate identification of the species source of processed tuberous roots marketed as Asparagi Radix can be achieved by recovering their complete plastomes from high-throughput sequencing reads and then comparing them with the reference plastome dataset generated in this study. The PCR-free molecular authentication method established in this study can serve as an efficient approach for preventing and controlling the collection and trade of tuberous roots from the nine medicinal *Asparagus* species that are not the botanic origins of Asparagi Radix.

Admittedly, taxon sampling in this study did not comprehensively encompass all currently recognized species within the economically important genus Asparagus. Moreover, complete plastomes were not obtained from the processed tuberous roots of these target *Asparagus* species in this study. To rationally exploit and conserve medicinal *Asparagus* species, sustained efforts are necessary to expand and complete the existing reference plastome library for the genus, as well as to develop an efficient method for recovering complete plastomes from processed tuberous roots of *Asparagus* species by application of high throughput sequencing technologies. Such advancements will significantly contribute to enhancing the efficacy and safety assurance of Asparagi Radix products, while also facilitating the conservation and sustainable utilization of medicinal *Asparagus* species.

Conclusion

The present study developed a PCR-free molecular authentication method that enables to accurate identify *Asparagus cochinchinensis*, the botanical source of Asparagi Radix, as well as the nine congeneric species, namely *A. dauricus*, *A. filicinus*, *A. meioclados*, *A. lycopodineus*, *A. myriacanthus*, *A. officinalis*, *A. oligoclonos*, *A. schoberioides*, and *A. taliensis*, which are frequently harvested from the wild populations as the adulterants of Asparagi Radix. This objective was achieved by employing the genome skimming approach to generate complete plastomes for serving as molecular markers to carry out species identification. Based on extensive inter- and intra-specific sampling, comprehensive phylogenetic and genetic distance analyses demonstrated that complete plastomes can serve as the optimal molecular markers for accurately identifying these ten target *Asparagus* species, as the inter-specific genetic divergence of complete platomes aligns with taxonomic delineated species boundaries. Given the immense potential of the genome skimming approach in generating complete plastomes from processed plant products containing highly degraded genomic DNA as molecular markers for species identification [32, 38–40], the molecular authentication method developed in this study can serve as an effective tool for tracing the species origins of processed tuberous roots marketed as Asparagi Radix, thereby effectively detecting commonly used adulterants in this pharmaceutically important traditional Chinese medicine. In light of this, the development of the PCR-free molecular authentication method in this study will contribute to the quality control of pharmaceutical products derived from Asparagi Radix. Additionally, it will also facilitate the conservation efforts and optional exploitation of its adulterants, corresponding to the nine medicinal *Asparagus* species traditionally used as ethnomedicines.

Abbreviations

bp	Base pair
BP	Bootstrap percentage
BS	Bootstrap
CTAB	Cetyl trimethylammonium bromide
DNA	Deoxyribonucleic acid
Gbp	Giga base pairs
IR	Inverted repeat
ITS	Internal transcribed spacer of nuclear ribosomal DNA
LSC	Large single-copy
ML	Maximum Likelihood
rRNA	Ribosomal RNA
SSC	Small single copy
tRNA	Transfer RNA

Supplementary Information

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Supplementary Material 1

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Author contributions

XG, YJ, GZ, and WS conceived and designed the research framework. PX, and TW collected and analyzed the data. XG, PX, GZ, and YJ wrote the original

draft manuscript. JL, XZ, WS, and YJ revised and edited the final manuscript. All authors have read and agreed to the published version of the manuscript.

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Data availability
The sequences generated in this study are available at the NCBI GenBank database, with the accession numbers being presented in Table S1.

Declarations

Ethics approval and consent to participate
The study was carried out in compliance with relevant institutional, national, and international guidelines and legislation for plant ethics. Collection of all plant samples in this study completely followed the Regulations on the Protection of Wild Plants of the People's Republic of China and with the official permission from local forestry authorities. All the methods included in this study are in accordance with the relevant ethical code of conduct.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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