Pak. J. Bot., 57(6): 2409-2422, 2025. DOI: http://dx.doi.org/10.30848/PJB2025-6(43)

PHYLOGENETIC ANALYSIS AND IDENTIFICATION OF THE MEDICINAL PLANT *ASPARAGUS COCHINCHINENSIS* AND RELATED SPECIES (ASPARAGACEAE) USING DNA BARCODING MARKERS

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Abstract

The goal is to identify *Asparagus cochinenchinensis*, a significant medicinal plant, and related species using a universal DNA barcode. We evaluated the potential of three widely used regions-ITS, *trn*H-*psb*A, and *trn*D-*trn*F, as candidate DNA barcodes to distinguish *A. cochinchinesis* from closely related species and to assess the phylogenetic relationships among 26 accessions representing 10 *Asparagus* species. The analysis included data on the success rate of PCR amplification, sequence alignment, identification efficiency, genetic distance between and within specific species, data distribution, DNA barcoding gap, and the conditions for maximum likelihood (ML) phylogenetic trees condition were all included in the analysis. In the barcoding of asparagus, ITS yielded the best results out of the three loci tested and that the combination of ITS+*trn*D-*trn*F+*trn*H-*psb*A shows significant potential as a robust, conventional DNA barcode for *Asparagus* identification. Additionally, the ITS2 secondary structure provides another approach for identifying *Asparagus* species. These results might validate these methods and aid in accurate species identification for *Asparagus* medicinal plants.

Key words: Asparagaceae; *Asparagus cochinchinensis*; China; DNA barcoding markers; Phylogeny

Introduction

Asparagus L. (Linnaeus, 1753) is a large genus, belongs to the family Asparagaceae of Asparagale (APG III 2009), that contains over 200 species (Judd, 1997; Govearts, 1995). Thirty-one species are included in the flora of China, 15 of which are endemic, and two of which are introduced (Chen & Tamanian, 2000; Mousavizadeh *et al*., 2021). The genus is widely distributed across the old-world continents (Dahlgren & Clifford, 1985; Kubitzki, 1998), and it has been claimed that it originated in Africa, particularly South Africa and surrounding areas (Demissew, 2008), from which it spread rapidly through diversification and dispersal throughout Asia and Europe (Kubota *et al*., 2012). Many species in this genus are significant for both ecology and the economy due to their use as food (*A. acutifolius*, *A. albus*, *A. maritimus* and *A. officinalis*), medicine (*A. adscendens*, *A. racemosus*, *A. cochinchinensis*, and *A. verticillatus*) and ornamental purposes (*A. asparagoides*, *A. densiflorus*, *A. plumosus*, *A. setaceus* and *A. virgatus*) (Singh, 2016; Li *et al*., 2020; Idrees *et al*., 2021). The infrageneric classification classified *Asparagus* into three subgenera: *Asparagus*, *Myrsiphyllum* Willd (1808), and *Protasparagus* Oberm (1983) (Clifford & Conran, 1987).

Although all species in the subgenus *Asparagu*s are dioecious, while the other two subgenera are all hermaphrodites, with variances in floral morphology and ovule count (Fukuda *et al*., 2005). The genus *Asparagus* has a variety of ploidy levels (including 2, 4, 6, 8, 10, and 12x) with a fundamental chromosomal number of x = 10. Some species in this genus have also been seen to exhibit intraspecific ploidy variation (Mousavizadeh *et al*., 2016). Phylogenetic study of DNA sequence data (Castro *et al*., 2013; Mousavizadeh *et al*., 2021) supported their monophyletic origin, where polyploidy and sexual dimorphism acting as the primary driving forces of evolution, and dioecious species evolving from hermaphrodite species.

Asparagus cochinchinensis is found in China, Japan, Korea, Vietnam, and other temperate and tropical countries (Pahwa *et al*., 2022). It ranks as one of the oldest and most frequently employed traditional herbal treatments, and reports of its clinical therapeutic value have come via many countries (Wang *et al*., 2019). *A. cochinchinensis* was originally mentioned as a traditional Chinese medicine (TCM) in Shennong's Classic of Materia Medica, the first Chinese medicinal classic text (written more than 2000 years ago during the Dong Han Dynasty). The prolonged use and clinical investigations have proven its effectiveness as a treatment. Later, it was included in many other well-known books on Chinese herbs, including "Yao Xing Lun" (published during the Tang Dynasty, 618–907 A.D.) and "Ming Yi Bie Lu" (published during the Wei and Jin Dynasty, 220–420 A.D.). Furthermore, it was first included as a therapeutic traditional Chinese medicine (TCM) in the Pharmacopoeia of the People's Republic of China in 1977, where it remained until the most recent edition, published in 2020. Dried *A. cochinchinensis* roots have been used for millennia to treat inflammatory illnesses, constipation, asthma, coughing, and thrombosis (Pharmacopoeia of The People's Republic of China, 2010). The traditional Chinese prescription of this herb (Qisheng pill) contains 114 chemical components, including diosgenin, methyl protodioscin, ferroic acid, total saponin, and others, which can lower inflammation, regulate intestinal dysfunction, and diminish the effects of Alzheimer's disease (Xiong *et al*., 2022). In addition, *A.*

cochinchinensis-containing herbal formula water decoction is effective in treating intestinal ailments, specifically asthma and allergic airway inflammation (Luo *et al*., 2020). This species is a common ingredient in many traditional formulae that are frequently used in clinics, greatly improving patient outcomes in China and other traditional Asian medical systems. Numerous commercial applications of *A. cochinchinensi*s exist, encompassing food, cosmetics, and health items (Safriani *et al*., 2022). It is frequently used as a nutritional or dietary supplement (Siand *et al*., 2015), as a raw ingredient for fermentation and winemaking (Topolsk *et al*., 2021), and in whitening and anti-aging cosmetics. Therefore, its great potential and numerous development opportunities are worth exploring.

Due to a scarcity of dried roots of *A. cochinchinensis*, various similar species are commonly utilized and sold in China because of their comparable morphological traits. These include the dried roots of *A. racemosus*, *A. meioclados*, *A. sichianicus*, *A. lycopodineus*, and *A. schoberioides*. Furthermore, species (including *A. cochinchinensi*s) within the same genus or family have comparable physical traits and are frequently misidentified, resulting in avoidable economic losses. Hence, there is an urgent need to develop an effective and rapid method for distinguishing *A. cochinchinensis* from its closely related species. Furthermore, the complete chloroplast genome of 14 *Asparagus* species are currently available in GenBank (NCBI: National Centre for Biotechnology Information): *A. subscandens* (NC_086747.1, PP180014.1); *A. officinalis* (NC_034777.1); *A. falcatus* (NC_086746.1); *A. setaceus* (NC_047458.1); *A. taliensis* (NC_088049.1); *A. meiocladus* (NC 080871.1); *A. densiflorus* (MT740250.1), *A. cochinchinensis* (NC_060472.1); *A. dauricus* (MT712151.1), *A. manitus* (NC_080870.1); *A. racemosus* (NC_047472.1); *A. schoberioides* (NC_035969.1), *A. lycopodineus* (NC_085423.1, OR8264.1) and *A. filicinus* (NC_046783.1). This represents a small percentage of the species in the genus, leaving a significant information gap in *Asparagus* molecular research. Unfortunately, the genetic history, phylogenetic relationships, and sources of variation for Chinese *Asparagus* species and accessions are presently not systematically documented, limiting the optimal application of *Asparagus* germplasm resources for hybrid breeding (Chen *et al*., 2020; Zhou *et al*., 2012).

DNA barcoding has become an emerging identification method that uses specific DNA sequence data to figure out and recognize species (Hebert *et al*., 2003). Plant identification has been shown to benefit from DNA barcoding, which is also helpful in ecological and evolutionary research (Newmaster *et al*., 2013). A number of gene regions, and combinations thereof, have recently been proposed for use in plants, despite the fact that the challenges of plant barcoding have been discussed (Fazekas *et al*., 2009). These include the nuclear internal transcribed spacer (ITS) region, plastid coding regions (e.g. *matK*, *rpoB*, *rpoC1*, and *rbcL*), and intergenic spacers regions (e.g. *atpF–atpH, psbK–psbI,* and *trnH–psbA*) (Chase *et al*., 2005; Kress and Erickson 2007). A DNA sequence cannot be used to identify species unless it has unique alterations that allow for the differentiation of closely related species. The ITS region should be included in core barcodes when direct sequencing is feasible since, according to the China Plant BOL Group, it has a better discriminating value than plastid barcodes (CBOL Plant Working Group 2009; Cowan *et al*., 2006). They recommended adopting ITS2 as a backup because of its conserved sequence features, which lessen amplification and sequencing issues, in order to circumvent the challenges of sequencing the complete ITS (CBOL Plant Working Group 2009). Therefore, in the current paper, we tested three potential DNA barcode regions (ITS, *trnD-trnF*, and *trnH–psbA*) as well as ITS2 to determine their ability to distinguish *A. cochinchinesis* from its closely related species as well as to assess the phylogenetic relationships between 26 accessions representing 10 species, seven of which are medicinally important in China. Hence, to (1) clarify the botanical sources of *A. cochinchinesis* and to distinguish the authentic herb from its closely related species. (2) clarify the molecular phylogenetic relationships of Chinese *Asparagus* species.

Material and Methods

Plant materials: Plant specimens (Table 1) from 26 accessions representing 10 species, including seven important medicinal herbs, were collected between 2022 and 2024 from diverse areas in southwest China. Every species was cultivated in Neijiang Normal University's greenhouse. The freshly picked leaves were collected and then dried with silica gel. The Neijiang Normal University Herbarium now houses the voucher samples. Table 1 lists the GenBank accession number for each primer.

DNA extraction, amplification, and sequencing: Using a modified cetyltrimethylammonium bromide (CTAB) technique supplemented with 1% PVP, total genomic DNA was isolated from fresh leaves or silica-gel dried leaves (Zhang *et al*., 2024). Qubit (Invitrogen) or 0.8% agarose gel electrophoresis was used to assess DNA purity. In a T100MT PCR Thermal cycler (Bio-Rad), three barcodes (ITS) were amplified by PCR using a 50 µL tube containing 25 µl of Taq PCR mastermix, 2 µl of each forward and reverse primer (10 mmol/L), 2 µl of genomic DNA (20–100 ng), and 19 µl of sterile water. Table 2 lists the reaction conditions and primers utilised in this investigation. The PCR product was sent to Tsingke Biological Technology Co., Ltd. (Chengdu, China) for sequencing after being electrophoresed on 1% Agarose in 1xTAE buffer, stained with ethidium bromide, and visualized under a UV lamp.

DNA barcode and DNA polymorphism analysis: The BioEdit software version 7.0.5.3 (Hall, 1999) was used to edit and trim the retrieved DNA sequences for each region. To confirm their similarity with sequences in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/ BLAST/), BLAST searches were then performed. Each gene's alignment was inferred using MAFFT v. 7.0 (Katoh and Standley 2013), and it was subsequently manually modified in MEGA version 11 (Tamura *et al*., 2013). The nuclear and concatenated plastid datasets were examined both alone and together.

In order to ascertain the average AT% and GC% nucleotide compositions for the three barcode primers, the resulting sequences were further analysed. Using DNAsp software, a thorough analysis of genetic diversity was

carried out (Librado and Rozas 2009), with particular attention paid to nucleotide diversity and segregating sites for each region in 26 sequences. The Tajima's D test was then utilized to evaluate departures from neutrality, yielding important details regarding the evolutionary history and demographic background of the examined *Asparagus* species.

The phylogenies were reconstructed using Maximum Likelihood (ML) approaches (Nei 7 Kumar, 2000). In Modeltest 2.1.10, DNA substitution models were selected for gene partitions based on the Bayesian information criterion (BIC). Genomic connections between barcode sequences were calculated using the Kimura 2-parameter (K2P) proximity model and MEGA version 11 (Tamura *et al*., 2013). The methods outlined by Chen *et al*., (2010) and Meyer & Paulay (2005) were used to graph the barcoding gaps through contrasting the spectrum of intra- and inter-specific divergences of each barcode. The Automatic Barcode Gap Discovery (ABGD) tool (Puillandre *et al*., 2012) was used to assess the barcodes' ability to be identified; all other settings were set to their default values.

Secondary structure analysis: We selected one haplotype for secondary structure prediction using the ITS2 Workbench (http://its2.bioapps.biozentrum.uni-wuerzburg.de/) in order to evaluate the usefulness of ITS2 secondary structure for the identification of *A. cochinchinesnsis* and closely related species (Koetschan *et al*., 2012).

Table 1. *Asparagus* species, collection, and voucher specimen information.

Species	Collected Place	Voucher number	GenBank Accession No. ITS; *trn*H-*psb*A; *trn*D-*trn*F
A. cochinchinensis 1	Neijiang, Sichuan	NJ001AC	PP947271; PQ439125; PQ439151
A. cochinchinensis 2	Neijiang, Sichuan	NJ002AC	PP947272; PQ439126; PQ439152
A. cochinchinensis 3	Neijiang, Sichuan	NJ003AC	PP947273; PQ439127; PQ439153
A. cochinchinensis 4	Neijiang, Sichuan	NJ004AC	PP947274; PQ439128; PQ439154
A. cochinchinensis 5	Neijiang, Sichuan	NJ005AC	PP947275; PQ439129; PQ439155
A. cochinchinensis 6	Wuxi, Chongqing	CQ001AC	PP947276; PQ439130; PQ439156
A. cochinchinensis 7	Luzou, Sichuan	LZ001AC	PP947277; PQ439131; PQ439157
A. cochinchinensis 8	Yibin, Sichuan	YB001AC	PP947278; PQ439132; PQ439158
A. densiflorus 1	Neijiang, Sichuan	NJ001AD	PP947279; PQ439133; PQ439159
A. densiflorus 2	Neijiang, Sichuan	NJ002AD	PP947280; PQ439134; PQ439160
A. densiflorus 3	Neijiang, Sichuan	NJ003AD	PP947281; PQ439135; PQ439161
A. filicinus	Liangshan, Sichuan	LS001AF	PP947282; PQ439136; PQ439162
A.lycopodineus 1	Yibin, Sichuan	YB001AL	PP947283; PQ439137; PQ439163
A.lycopodineus 2	Leshan, Sichuan	LS001AL	PP947284; PQ439138; PQ439164
A.lycopodineus 3	Leshan, Sichuan	LS002AL	PP947285; PQ439139; PQ439165
A. mairei	Liangshan, Sichuan	LS001AM	PP947286; PQ439140; PQ439166
A. officinalis	Neijiang, Sichuan	NJ001AO	PP947287; PQ439141; PQ439167
A. setaceus	Neijiang, Sichuan	NJ001AS	PP947288; PQ439142; PQ439168
A. taliensis 1	Neijiang, Sichuan	NJ001AT	PP947289; PQ439143; PQ439169
A. taliensis 2	Bijie, Guizhou	BJ001AT	PP947290; PQ439144; PQ439170
A. taliensis 3	Bijie, Guizhou	BJ002AT	PP947291; PQ439145; PQ439171
A. taliensis 4	Luzou, Sichuan	LZ001AT	PP947292; PQ439146; PQ439172
A. taliensis 5	Zunyi, Guizhou	ZY001AT	PP947293; PQ439147; PQ439173
A. yanyuanensis	Liangshan, Sichuan	LS001AY	PP947294; PQ439148; PQ439174
*A. sp*1	Neijiang, Sichuan	NJ001un	PP947295; PQ439149; PQ439175
*A. sp*2	Luzou, Sichuan	LS001un	PP947296; PQ439150; PQ439176
Cordyline	-	GenBank	KY908606; JX574446; JX574655
Maianthemum	-	GenBank	KY908535; KX375110; JX574657

Table 2. PCR primers and conditions for barcode sequence amplification.

Gene and region	Primer names	Primer sequence 5′–3′	Reaction condition
ITS	ITS1	CCGGTGAAGTGTTCGGATCGC	94°C for four minutes, 94°C for thirty seconds, 55°C for 30 seconds, 72°C for 1 minute, 35 cycles, then 72°C for 7 minutes
	ITS2	AGCTGGGCTATTCCCGGTTCG	
*trn*H-*psb*A	*trn*H	CGCGCATGGTGG ATTCACAATCC	94°C for five minutes, 94°C for thirty seconds, 55–64.1°C for 30 seconds, 72°C for 1.5 minutes, 35 cycles, then 72°C for 10 minutes
	*psb*A	GTTATGCATGAACGTAATGCTC	
*trn*D-*trn*F	*trn*D	ACCAATTGAACTACAATCCC	94°C for five minutes, 94°C for thirty seconds, 55–64.1°C for 30 seconds, 72°C for 1.5 minutes, 35 cycles, then 72°C for 10 minutes
	*trn*F	CTACCACTGAGTTAAAAGGG	

Table 3. DNA barcoding using single and combination barcodes in *Asparagus*.

DNA barcodes	Total accessions	Sequenced accessions (%)	Sequenced species (%)	Aligned length (bp)	Conserved sites	Variable sites (bp)	Parism-Infor Sites	T (%)	C (%)	A (%)	G (%)	AT (%)	GC (%)
ITS	26	26(100)	10(100)	805	254	535	361	21.1	31.7	18.1	29.1	39.2	60.8
*trn*H-*psb*A	26	26(100)	10(100)	623	267	343	18	30	17.2	34.9	17.9	64.9	35.1
*trn*D-*trn*F	26	26(100)	10(100)	938	751	161	70	32	18.5	31.6	17.9	63.6	36.4
*trn*H-*psb*A+*trn*D-*trn*F	26	26(100)	10(100)	1560	938	588	57	31.3	17.9	33	17.9	64.3	35.8
ITS+*trn*H-*psb*A	26	26(100)	10(100)	1428	521	878	379	24.9	25.4	25.4	25.3	50.3	50.7
ITS+*trn*D-*trn*F	26	26(100)	10(100)	1742	1005	695	430	27	24.6	25.4	23.1	52.4	47.7
ITS+*trn*H-*psb*A+*trn*D-*trn*F	26	26(100)	10(100)	2365	1192	1123	418	27.7	22.7	27.8	21.8	55.5	44.5

Results

Success rate of PCR amplification and sequence features: In this investigation, all *Asparagus* species samples had 100% PCR amplification and sequencing success rates for ITS, *trn*H-*psb*A, and *trn*D-*trn*F sequences (Table 3). Based on multiple alignments of all sequences in each region, *trn*D-*trn*F had the largest length (938 bp), while *trn*H-*psb*A had the shortest length (623 bp). For individual barcode regions, the ITS region had a high number of variable sites 535 and 361 parsimony informative sites (Table 3) whereas *trn*H-*psb*A and *trn*D-*trn*F regions had 343 and 161 variable sites and 18 and 70 parsimony informative sites, respectively. ITS had the highest mean GC content (60.8%), followed by *trnD-trnF* (36.4%), while *trn*H-*psb*A had the lowest mean GC ratio (35.1%). The mean AT ratio in ITS was 39.2 %, which was lower than the AT ratios in *trn*H-*psb*A (64.9 %) and *trn*D-*trn*F (63.6%). In the case of combined barcode regions: ITS+*trn*H-*psb*A+*trn*D-*trn*F had a high number of variable sites 1123 and 418 parsimony informative sites but had the lowest mean GC ratio (44.5%) and the highest mean AT ratio (55.5%) when compared to ITS+*trn*H-*psb*A, ITS+*trn*D-*trn*F, and *trn*H-*psb*A+*trn*D-*trn*F.

DNA polymorphism analysis: Table 4 contains a detailed breakdown of DNA polymorphisms in Asparagus species, encompassing 26 variants that correspond to three barcode areas. Regarding specific barcode regions: With 316 segregating sites found by ITS, the average number of nucleotide differences (k) was 45.7261, and the nucleotide diversity (π) was a comparatively high 0.06238. *trn*D-*trn*F and *trn*H-*psb*A had 61 and 9 segregating sites, respectively, resulting in slightly reduced nucleotide diversity (π) of 0.01316 and 0.00204 as well as 11.274 and 0.905 average number of nucleotide differences (k). The greatest number of haplotypes was created by *trn*D-*trn*F (23), followed by ITS (22) and *trn*H-*psb*A (6). For combined barcode regions: ITS+*trn*H-*psb*A+*trn*D-*trn*F and ITS+*trn*D-*trn*F exhibited 386 and 377 segregating sites, but reduced nucleotide diversity (π) of 0.02848 and 0.03585 as compared to ITS+*trn*H-*psb*A (0.03965), respectively.

Phylogenetic analysis

BLASTn analysis: The species found using the three barcode regions in the BLAST search are listed in Table 5, along with their repository numbers, and are contrasted with taxonomic identification. The results show the percentages of the subject species' highest level of similarity; most samples yielded sequences that were categorized as best hits (98–100%). Based on the efficiency of the three markers in BLAST searches, *ITS, trnH-psbA,* and *trnD-trnF* obtained exceptional 98-100% accuracy at the genus and species levels, demonstrating their potential to capture broader taxonomic groups. *A. cochinchinensis* samples 1-8 exhibited 100% resemblance to the same species and with a similarity of 98-100%. Species such as *A. densiflorus* 3 (ITS & *trn*D-*trn*F), *A. lycopodineus* 1 (*trn*D-*trn*F), *A. mairei* (ITS, *trnH-psb*A and *trn*D-*trn*F), *A. yanyuanensis* (ITS, *trn*H-*psb*A and *trn*D-*trn*F), and undescribed species *A. sp*1 and *A. sp*2 showed exhibited 81-100% similarity with other species.

Table 4. Summary analysis of DNA polymorphism in *Asparagus* species based on DNA barcode regions.

DNA barcodes	S	Eta	H	Hd	π	k	p
ITS	316	407	22	0.985 ± 0.016	0.06238	45.7261	$p<0.01$
*trn*H-*psb*A	9	10	6	0.468 ± 0.012	0.00204	0.905	$p<0.05$
*trn*D-*trn*F	61	64	23	0.991 ± 0.013	0.01316	11.274	$p>0.10$
*trn*H-*psb*A + *trn*D-*trn*F	70	74	24	0.994 ± 0.013	0.00937	12.178	$p>0.10$
ITS + *trn*H-*psb*A	325	417	22	0.985 ± 0.016	0.03965	46.631	$p<0.01$
ITS + *trn*D-*trn*F	377	471	25	0.997 ± 0.012	0.03585	57.000	$p<0.05$
ITS + *trn*H-*psb*A + *trn*D-*trn*F	386	481	25	0.997 ± 0.012	0.02848	57.905	$p<0.05$

S=Number of segregating sites; Eta=total number of Mutations; H=Haplotype; Hd= Haplotype diversity; π= nucleotide diversity; k=Average number of nucleotide differences; p=Statistical significant.

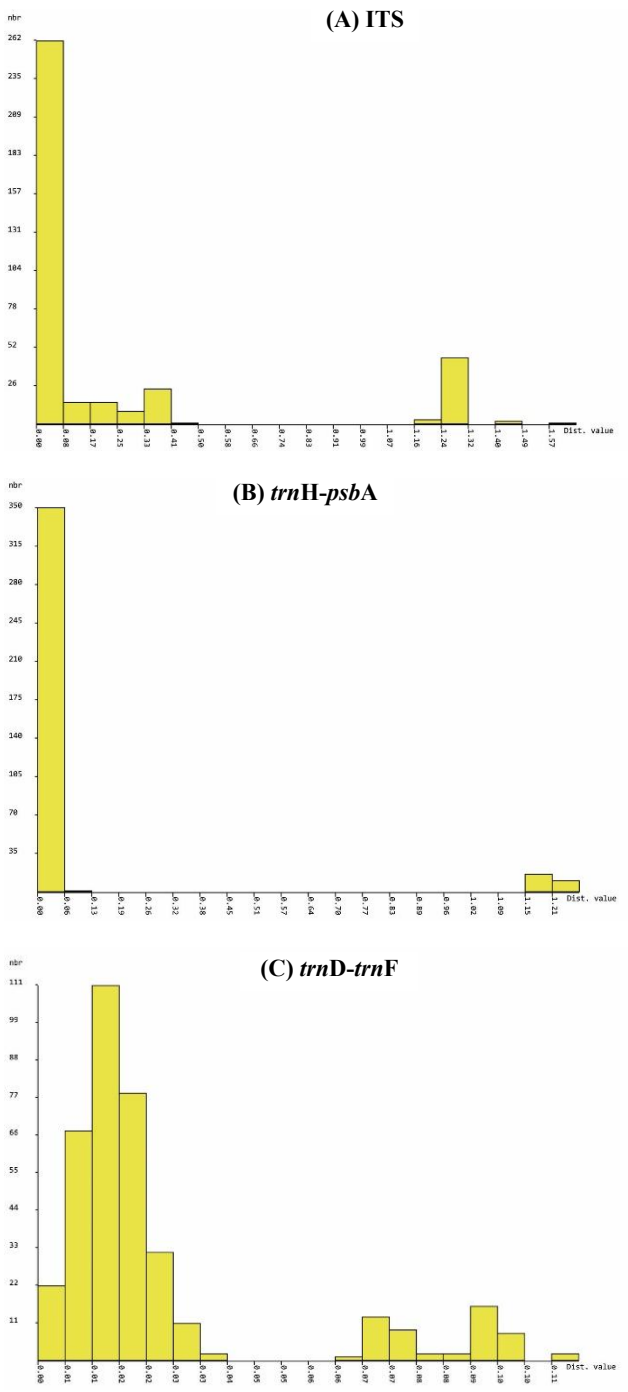

Fig. 1. Relative distribution of intraspecific and interspecific distances for three barcodes regions. (A) ITS, (B) *trn*H-*psb*A, (C) *trn*D-*trn*F. The intraspecific distance is shown by the left bar, while the interspecific distance is shown by the right bar. The interval-based Kimura-2-parameter distances are represented by the x-axes, while the proportion of occurrences is represented by the y-axes.

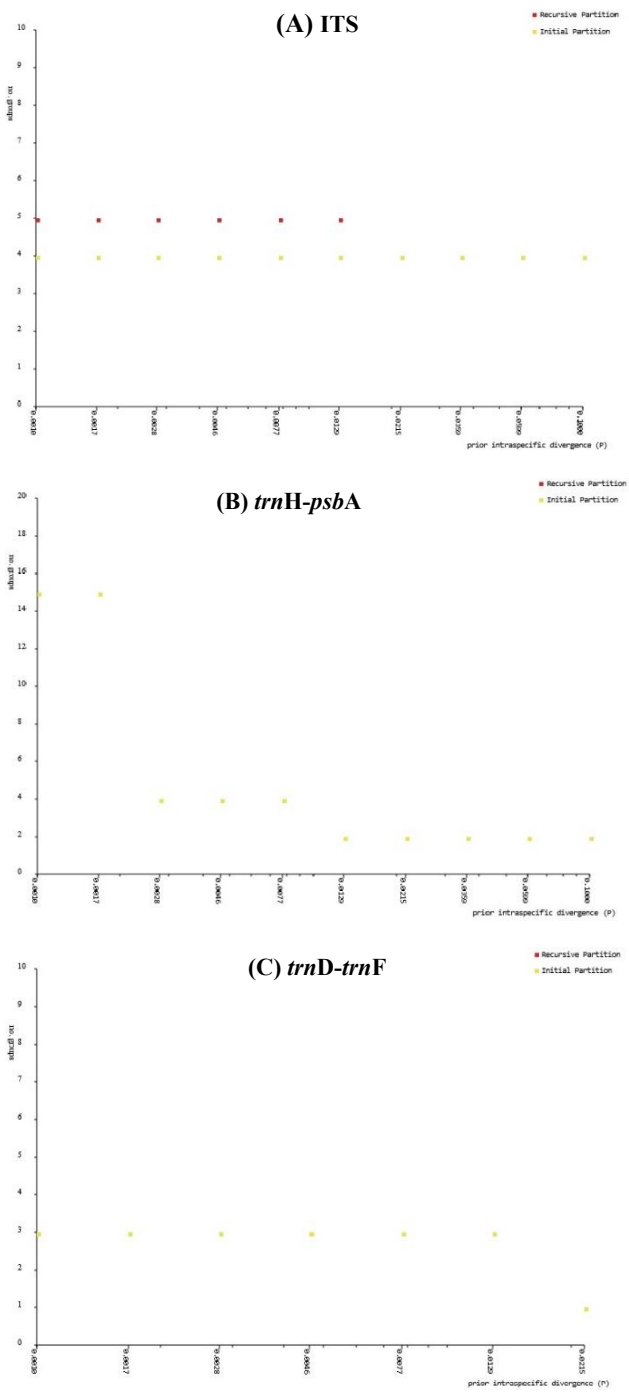

Fig. 2. Automatic classification of barcode sequences by the ABGD program. (A) ITS, (B) *trn*H-*psb*A, (C) *trn*D-*trn*F. The initial partition is represented by the yellow dots, and the recursive partition by the red dots. The number of groups is represented by the y-axes, whereas the x-axes are related to previous intraspecific divergence.

Table 5. *Aparagus* species and the percentage of similarity with the closest species in the GenBank based on DNA barcode regions.

Species	Accession No. ITS psbA trnD	Per. Identity (%)	E-value	Cover (%)	Accession No. of the best match
	ITS	98.56	0.0	100	OQ857357.1
A. cochinchinensis 1	*trn*H-*psb*A	100	0.0	100	GU135434.2
	*trn*D-*trn*F	99.55	0.0	99	JX574474.1
	ITS	99.35	0.0	100	OQ857357.1
A. cochinchinensis 2	*trn*H-*psb*A	99.83	0.0	99	KU198257.1
	*trn*D-*trn*F	98.76	0.0	99	JX574474.1
	ITS	99.48	0.0	100	OQ857357.1
A. cochinchinensis 3	*trn*H-*psb*A	100	0.0	100	KU198257.1
	*trn*D-*trn*F	98.98	0.0	99	JX574474.1
	ITS	98.56	0.0	100	OQ857357.1
A. cochinchinensis 4	*trn*H-*psb*A	99.50	0.0	100	KU198257.1
	*trn*D-*trn*F	99.54	0.0	99	JX574474.1
	ITS	98.56	0.0	100	OQ857357.1
A. cochinchinensis 5	*trn*H-*psb*A	99.50	0.0	100	KU198257.1
	*trn*D-*trn*F	99.66	0.0	99	JX574474.1
	ITS	99.35	0.0	100	OQ857361.1
A. cochinchinensis 6	*trn*H-*psb*A	99.33	0.0	100	KU198257.1
	*trn*D-*trn*F	99.77	0.0	99	PP505531.1
	ITS	99.48	0.0	100	OQ857361.1
A. cochinchinensis 7	*trn*H-*psb*A	99.50	0.0	100	KU198257.1
	*trn*D-*trn*F	98.41	0.0	99	PP505531.1
	ITS	99.48	0.0	100	OQ857361.1
A. cochinchinensis 8	*trn*H-*psb*A	99.79	0.0	100	KC704257.1
	*trn*D-*trn*F	99.43	0.0	99	PP505531.1
	ITS	98.83	0.0	100	OQ857341.1
A. densiflorus 1	*trn*H-*psb*A	100.00	0.0	100	HM990141.1
	*trn*D-*trn*F	97.98	0.0	99	MZ337395.1
	ITS	99.09	0.0	100	OQ857341.1
A. densiflorus 2	*trn*H-*psb*A	100	0.0	100	HM990141.1
	*trn*D-*trn*F	98.77	0.0	99	JX574523.1
	ITS	99.35	0.0	100	OQ857364.1
A. densiflorus 3	*trn* H-*psb*A	99.66	0.0	100	HM990141.1
	*trn*D-*trn*F	99.32	0.0	99	NC_088049.1
	ITS	99.48	0.0	100	OQ857344.1
A. filicinus	*trn*H-*psb*A	99.78	0.0	99	NC_046783.1
	*trn*D-*trn*F	99.77	0.0	99	OR807823.1
	ITS	99.09	0.0	100	OQ857344.1
A.lycopodineus 1	*trn*H-*psb*A	95.29	0.0	100	HM990124.1
	*trn*D-*trn*F	99.43	0.0	99	NC_086747.1

Table 5. (Cont'd.).

Species	Accession No. ITS psbA trnD	Per. Identity (%)	E-value	Cover (%)	Accession No. of the best match
	ITS	99.61	0.0	100	OQ857344.1
A.lycopodineus 2	*trn*H-*psb*A	99.66	0.0	100	GU135434.2
	*trn*D-*trn*F	99.66	0.0	99	PP505533.1
	ITS	99.48	0.0	100	OQ857344.1
A.lycopodineus 3	*trn*H-*psb*A	99.16	0.0	100	GU135434.2
	*trn*D-*trn*F	99.66	0.0	99	NC_086747.1
	ITS	88.01	1e-96	99	OQ857368.1
A. mairei	*trn*H-*psb*A	99.33	0.0	100	GU135434.2
	*trn*D-*trn*F	96.58	0.0	100	PP505532.1
	ITS	98.96	0.0	100	OQ857367.1
A. officinalis	*trn*H-*psb*A	99.83	0.0	100	HM990135.1
	*trn*D-*trn*F	100.00	0.0	99	LN896356.1
	ITS	99.22%	0.0	100%	OQ857334.1
A. setaceus	*trn*H-*psb*A	99.50%	0.0	100%	HM990138.1
	*trn*D-*trn*F	98.78%	0.0	99%	NC_047458.1
	ITS	99.74%	0.0	100%	OQ857364.1
A. taliensis 1	*trn*H-*psb*A	100%	0.0	100%	GU135434.2
	*trn*D-*trn*F	99.55%	0.0	99%	NC_088049.1
	ITS	99.61%	0.0	100%	OQ857364.1
A. taliensis 2	*trn*H-*psb*A	99.83%	0.0	100%	GU135434.2
	*trn*D-*trn*F	99.55%	0.0	99%	NC_088049.1
	ITS	99.61%	0.0	100%	OQ857360.1
A. taliensis 3	*trn*H-*psb*A	99.83%	0.0	100%	GU135434.2
	*trn*D-*trn*F	98.76%	0.0	99%	NC_088049.1
	ITS	97.79%	0.0	100%	OQ857364.1
A. taliensis 4	*trn*H-*psb*A	100%	0.0	100%	GU135434.2
	*trn*D-*trn*F	99.32%	0.0	99%	NC_088049.1
	ITS	99.09%	0.0	100%	OQ857364.1
A. taliensis 5	*trn*H-*psb*A	100%	0.0	100%	GU135434.2
	*trn*D-*trn*F	99.77%	0.0	99%	NC_088049.1
	ITS	99.74%	0.0	100%	OQ857364.1
A. yanyuanensis	*trn*H-*psb*A	100%	0.0	100%	HM990141.1
	*trn*D-*trn*F	99.66%	0.0	99%	NC_088049.1
	ITS	81.73%	1e-167	97%	OQ857361.1
*A. sp*1	*trn*H-*psb*A	99.57%	0.0	98%	NC_035969.1
	*trn*D-*trn*F	99.54%	0.0	99%	JX574517.1
	ITS	99.22%	0.0	100%	OQ857341.1
*A. sp*2	*trn*H-*psb*A	100%	0.0	100%	HM990141.1
	*trn*D-*trn*F	99.66%	0.0	99%	NC_086746.1

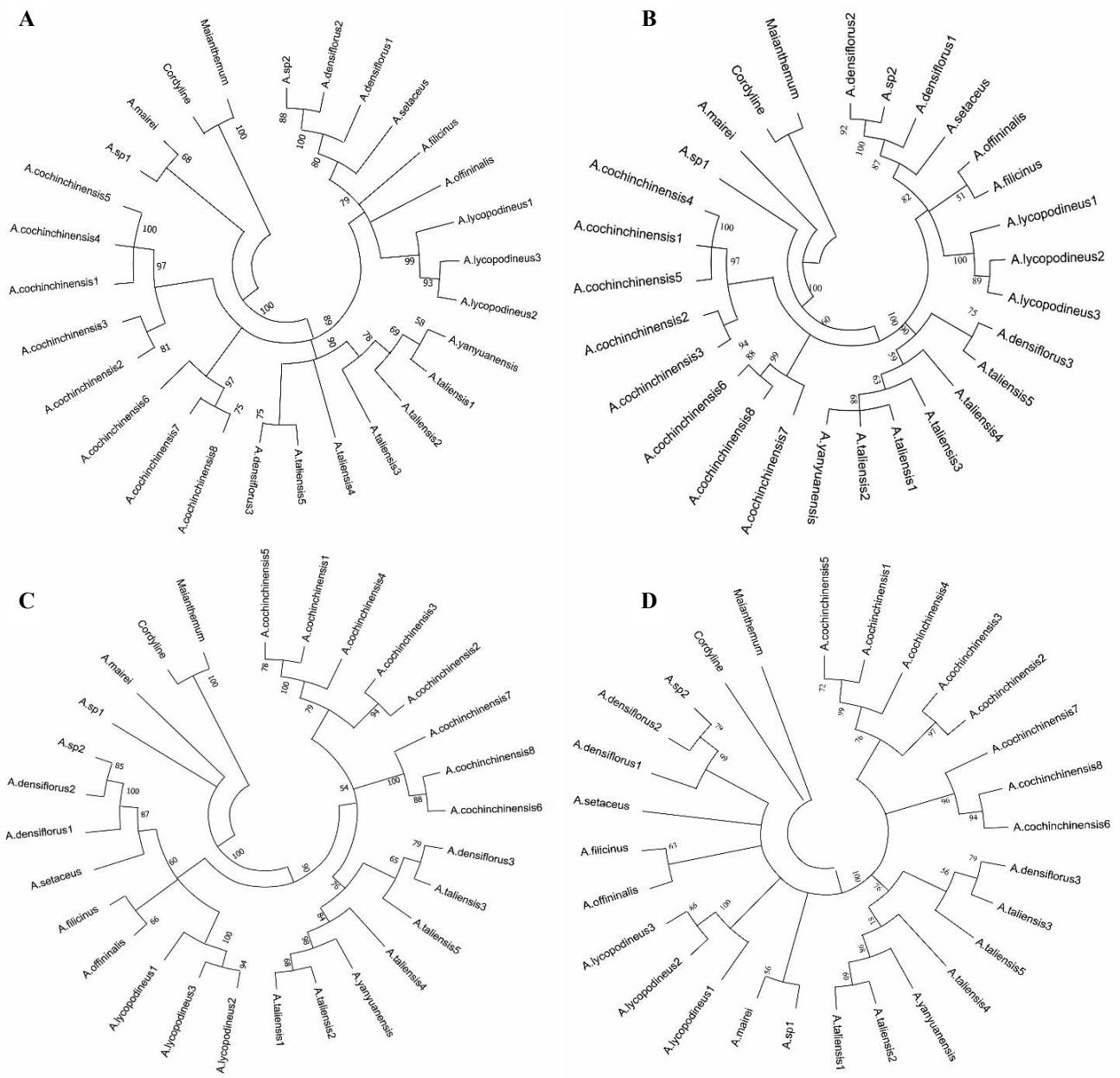

Fig. 3. Maximum likelihood (ML) tree. A. ITS; B. ITS+*trn*H-*psb*A; C. ITS+*trn*D-*trn*F; D. ITS+*trn*D-*trn*F+*trn*H-*psb*A. Bootstrap values (>50%) displayed above the corresponding branches.

Distance-based method: The pairwise genetic distance matrix reveals important information about the genetic relationships between 26 accessions representing 10 *Asparagus* species. The average interspecific and intraspecific genetic distances of 26 K2P measurements were 0.050 (0-1.32)/0.048 (0-1.27) for ITS, 0.038 (0-1.004)/0.003 (0-0.097) for *trn*H-*psb*A and 0.003 (0.002-0.097)/0.002 (0-0.075) for *trn*D-*trn*F. The mean interspecific distances for the four combination markers and the three barcode sections were generally greater than the mean intraspecific distances. While ITS had the biggest barcoding gap, with more than ten times the intraspecific vs. interspecific distance, the combined (*trn*H-*psb*A+*trn*D-*trn*F) regions displayed the shortest and most similar inter- vs. intraspecific distance. The species of *Asparagus* had mean sequence divergences in the following order: ITS>ITS + *trn*H-*psb*A + *trn*D-*trn*F>ITS + *trn*H-*psb*A>*trn*H-*psb*A + *trn*D-*trn*F>*trn*H-*psb*A + *trn*D-*trn*F> ITS + *trn*D-*trn*F>*trn*D-*trn*F.

The ITS can create a "barcoding gap" (Fig. 1), but the other barcodes are unable to distinguish between intraspecific and interspecific distances, according to the relative distribution of intraspecific and interspecific distances for the three DNA barcode regions in ten Asparagus species (Fig. 1). The Automatic Barcode Gap Discovery (ABGD) tool was used to evaluate if various barcodes could identify species based on genetic distance (Fig. 2). According to the results, the program initially divided the ITS barcode regions into five groups and discovered a barcode gap distance of 0.0183%; for the *trn*H-*psb*A, the barcode region was separated into 15 groups and a barcode gap centred around 0.0183% of distance was found; and for the *trn*D-*trn*F regions, the barcode regions were separated into three groups and a barcode gap distance of 0.053% was found.

Phylogenetic trees: Using the maximum likelihood (ML) approach, phylogenetic trees were built for the three barcode regions and four tested combinations. The ML-generated topology showed the highest support values for the ITS region (Fig. 3A), recovering 96% of species clusters with bootstrap support ranging from 74% to 100%, followed by ITS+*trn*H-*psb*A (63% to 100%) (Fig. 3B), ITS+*trn*D-*trn*F (51% to 100%) (Fig. 3C) and ITS+*trn*D-*trn*F+*trn*H-*psb*A (50% to 100%) (Fig. 3D). Compared to cpDNA markers alone, *trn*D-*trn*F (5% to 86%), *trn*H-*psb*A (1% to 50%), and *trn*H-*psb*A+*trn*D-*trn*F (10% to 97%) (Supl. A) had the lowest support values and displayed the worst performance for accurate identification and the highest percentage of wrong identifications. Undetermined verification rates were relatively low (<9%) for most individual locations and permutations. ITS in particular offered a more precise classification of the ten species than the other markers that were examined. The minimal bootstrap values in ITS were 74% to differentiate between species based on the phylogenetic tree, suggesting that the ITS barcode sequence provided better resolution for differentiating clusters that represented homogenous clades within each *Asparagus* species (Fig. 3A). *A. cochinchinensis*1-8 wild and cultivated species formed a group with 97% to 98% support value, *A. taliensis*1-5 species with 90% support value formed a group, *A. lycopodineus* samples 1-3 formed a group with 99% support value. The undescribed species *A. sp*1 formed a clade wih *A. maire*i with 67% support value. *A setaceus* formed a clade with *A. densiflorus* while *A. officinalus* and *A. filicinus* formed a trichome with *A. lycopodineus* clade.

Secondary structure analysis: The use of ITS2 secondary features in species identification was another topic we covered. The secondary structures of ITS2 for Asparagus species are shown in (Fig. 4). All of the ITS2 secondary structures of *A. cochinchinensis* and other species include a core ring and four helices that are similar to it: Helix I, II, III, and IV. However, depending on the number, size, location, and degree of angles from the spiral arm center, the secondary structures of ITS2 varied on four helices in the majority of *Asparagus* species. Compared to *A. cochinchinesis*: three species (*A. lycopodineus*, *A. yanyuanensis* and *A. taliensis*) had more stem-loops in helix I and III, but three other species such as *A. densiflorus*, *A. lycopodineus* and an undescribed species (*A. sp2*), were missing one stem-loops in the helix II. Furthermore, *A.sp*1 (an undescribed species) has a distinct secondary structure compared to the other species, with unpaired bases forming an outside stem-loop near the center ring. The stem loops near the end of helix III of *A. officinalis* and *A. filicinus* were larger than those of *A. cochinechinensis*. Thus, the secondary structure of ITS2 provides another approach for identifying *Asparagus* species. However, identical secondary structures were discovered in *A. densiflor*us and *A.sp1* (Fig. 4).

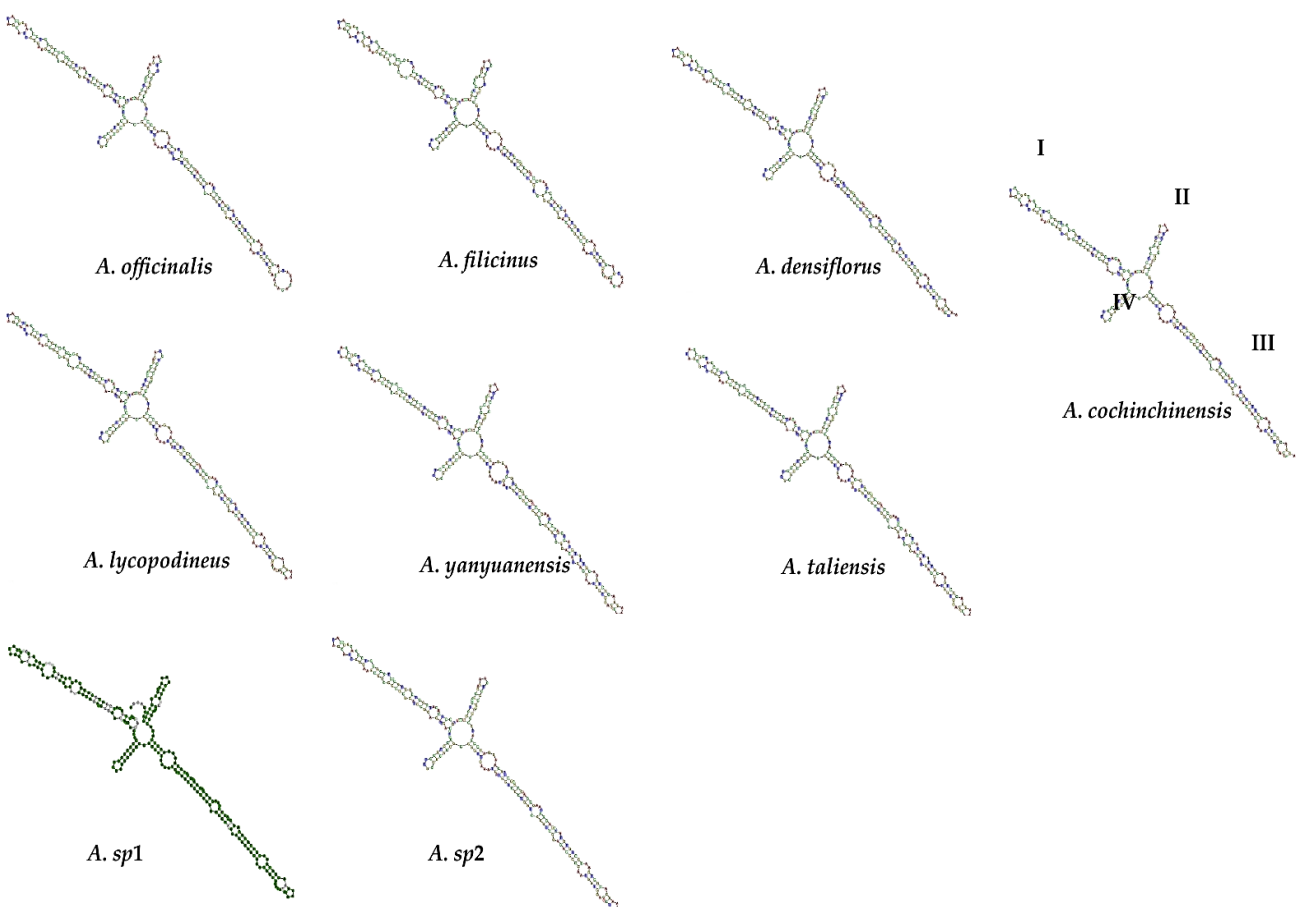

Fig. 4. The ITS2 secondary structure of *A. cochinchinensis* and other species.

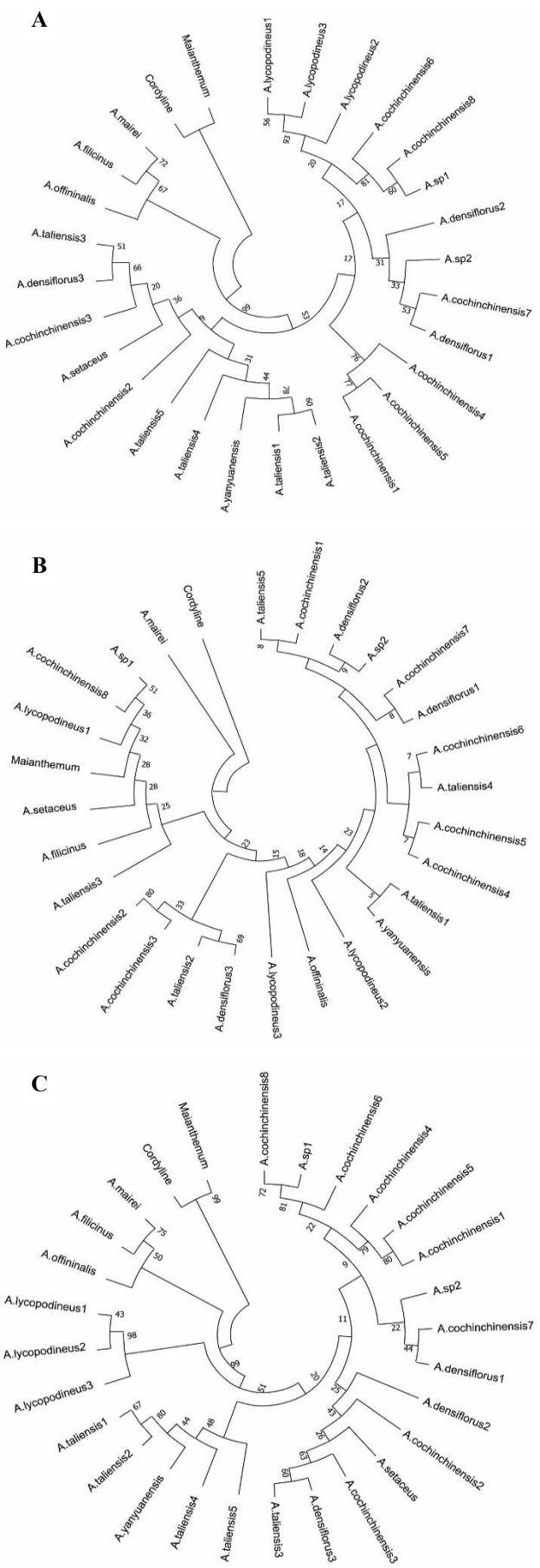

Fig. 5. Maximum likelihood (ML) tree. A.*trn*D-*trn*F; B. *trn*H-*psb*A; C. *trn*H-*psb*A+*trn*D-*trn*F. Bootstrap values are shown above the relevant branches.

Discussion

In China, the old methods of identifying herbs were based on their appearance, smell, or taste, and were carried out by skilled individuals. However, these methods were not always accurate, and it can be challenging to teach someone the necessary abilities. In recent times, precision technologies like liquid chromatography-mass spectrometry and microscopes have been employed to identify herbs by chemical analysis and microscopic approaches. Nevertheless, distinguishing between closely related species that have comparable physical traits or chemical profiles proved impossible due to the complexity and expense of these procedures. DNA-based authentication has been shown to be increasingly dependable and successful with the advancement of molecular techniques (Mishra *et al*., 2016). The species verification of several target herbal groups was accomplished by using DNA barcode markers (Liu *et al*., 2019; Lv *et al*., 2020).

The efficiency of three barcode loci in identifying medicinally important species of asparagus in China, namely in the Sichuan region, is being reported for the first time. The ITS is the major barcode area for the biological identification of *A. cochinenchinensis* and related species, according to the results of our study. This locus combines the features of molecular variation, intra- and inter-specific divergence patterns, BLASTn search, and the capacity to recover species-specific clades in phylogenetic trees. It is advised to include *trn*H-*psb*A and *trn*D-*trn*F as an additional locus since, in conjunction with ITS, the barcode combines nuclear and plastid genetic information, adding more dependability to the data set without enhancing the capacity to identify species taxonomically.

Amplification, success of sequencing, and maker characteristics: In our study, the three barcode regions (ITS, *trn*D-*trn*F and *trn*H-*psb*A) were easily amplified from 26 tested *Asparagus* samples and these regions had high sequencing success rates (100%) as well as high quality bidirectional sequences. A similar result, but with lower percentages, has been reported for other medicinal plants, with ITS and *trn*H-*psb*A PCR amplification and sequencing rates high enough to be termed as barcode loci (Tripathi *et al*., 2013; Bolson *et al*., 2015). Due to a high insertion/deletion rate, pseudogenes, repetitive loci, their length (> 1000 bp) and the complexity of bidirectional sequencing, some investigations rejected the loci *trn*H-*psb*A as the barcode (CBOL Plant Working Group 2009; Hollingsworth *et al*., 2009) as such, *trn*H-*psb*A sequence analysis requires manual correction, making comparing genera and species challenging. Nevertheless, we were able to achieve sequencing and PCR amplification success rates that were satisfactory (Table 3).

Recently, Norup *et al*., (2015) studied the phylogeny of *Asparagus* from regions outside of South Africa using one nuclear region (phytochrome C; PHYC), and three plastid regions (*trn*D-*trn*T, *trn*H-*psb*A, and *ndh*F). They concluded that ITS is quite difficult for phylogenetic purposes in *Asparagus*. This prevalent ITS pattern has been attributed to secondary structure development, endophytic fungal contamination, or large fluctuations in sequence length, numerous copies from paralogs, all of which result in low-quality sequences (Hollingsworth *et al*., 2009). When

compared to other plastid regions, our results showed that ITS provided a clearer classification of the species level phylogeny of Asian *Asparagus* species. Nevertheless, our results are compatible with prior studies in *Asparagus* (Saha *et al*., 2014; Akani *et al*., 2024), but not with recent studies (Norup *et al*., 2015; Yang *et al*., 2024). Furthermore, the ITS region has been proposed as a good supplement to core barcode loci, particularly in circumstances where the laboratory cost of synthesizing sequences was low or the very high species-discriminating rate compensated for the investment (Li *et al*., 2011; Vivas *et al*., 2014). More than 21,722 plant species have been identified using ITS as a universal barcode, and it is advised to utilize ITS to confirm the legitimacy of Chinese herbal medicine. Divergent paralogs are a common challenge in ITS, resulting in nonspecific amplifications and multiple bands on an agarose gel (Hollingsworth, 2011). However, the *Asparagus* species from China did not exhibit this issue.

Markers relative performance

BLASTn analysis: The 'best close match' test using BLASTn revealed that the three barcode regions (*ITS, trn*H-*psb*A and *trn*D-*trn*F*)* were the most effective in detecting *Asparagus* species. All sequences' BLASTn analysis showed that the samples had all been accurately recognised up to the species level based on the match between the DNA sequences of the plant samples and the scientific names obtained from the conspecific sequences stored in GenBank (Table 5). In a similar vein, ITS regions consistently exhibit the highest success rates for species identification (98–100%) according to BLASTn analysis, with the exception of the undescribed *A. sp*1, which displayed 81.73%. While *A. mairei* identification was strikingly low at the species level, accounting for only 88.01% of all collected samples. It is possible that the sequences are not available in the NCBI database, therefore they resemble the closest species. Whereas the chloroplast regions revealed 99% similarity to the diverse species in the genus. These findings support the current study's assertion that ITS can reliably identify both phylogenetically distant and closely related species (Akani *et al*., 2024; Gao *et al*., 2010; Saha *et al*., 2014; Wiegand, 2006).

Distance-based method: It has been suggested that the plastid regions and ITS regions be combined to create a universal barcode for land plants (CBOL Plant Working Group, 2009). Studies have demonstrated that this combination produces superior resolution for species discrimination (Kress and Erickson 2007; Cowan *et al*., 2006; Li *et al*., 2015) when compared to individual areas. In currently study, only 50% of the *Asparagus* species from China were accurately identified by this combination (ITS+*trn*D-*trn*F+*trn*H-*psb*A), which is less accurate than the separate regions (ITS, 75%; combined ITS+*trn*H-*psb*A (63% to 100%) and ITS+*trn*D-*trn*F (51% to 100%) and suggests that there is an unsuitable barcode gap. Similar poor efficacy was also shown by this combination in differentiating *Asparagus* species and they came to the additional conclusion that, in contrast to the limited resolution of plastid sequences, the nuclear region offers a good resolution for studying the species-level phylogeny of SW Asian *Asparagus* (Akani *et al*., 2024).

DNA barcoding for species identification is based on the theory that genetic distance within a species is much smaller than genetic distance between two species. It has been proposed that 2% is the threshold for species delimitation, and in general, the average genetic difference between two species (interspecific) is more than ten times larger than that within a species (intraspecific) (Hebert *et al*., 2003; Ward, 2009). The present investigation found that the mean interspecific distances were higher than the intraspecific distances for the three barcode regions and the four combination markers. In addition, as compared to other two barcode regions, ITS had higher interspecific distance and lower intraspecific distance in *Asparagus*. Although interspecific variation of the combined two barcode regions (*trn*H-*psb*A+*trn*D-*trn*F) in *Asparagus* was similar to its intraspecific variation. Moreover, the *trn*H-*psb*A or *trn*D-*trn*F regions, there were no significant differences between their interspecific and interspecific variations. With over 10 times the inter- vs. intraspecific distance, ITS had the largest barcoding gap, while the *trn*H-*psb*A+*trn*D-*trn*F regions had the smallest inter- vs. intraspecific distance. Furthermore, the ITS can create a "barcoding gap" (Fig. 1) based on the relative distribution of intraspecific and interspecific distances, while the other barcodes are unable to distinguish between intra- and interspecific distances.

Tree-based method: To study the evolutionary relationships of *A. cochinchinensis* within this genus, one nuclear ITS and two chloroplast DNA (cpDNA) sequences (*trn*H-*psb*A, and *trn*D-*trn*F) were sequenced. The Chinese *Asparagus* species were effectively differentiated in our study by the ITS region and the combinations ITS+*trn*H-*psb*A (63% to 100%), ITS+*trn*D-*trn*F (51% to 100%), and ITS+*trn*D-*trn*F+*trn*H-*psb*A (50% to 100%) with high support value (Fig. 3). The ITS region and its combinations shown comparable capacity in differentiating between species, even in closely related species, by identifying species-specific clusters across all taxa. The phylogenetic tree derived from the ITS sequence has a higher resolution than those obtained from cpDNA *trn*H-*psb*A and *trn*D-*trn*F sequences (Suppl. A); this is because it can distinguish the species with the closest relationships even within a subclade, and it was further determined that Eurasian species are monophyletic. Our findings agree with the earlier research conducted by Saha *et al*., (2014), and they concluded that compared to cpDNA *trn*L intron sequences, the phylogenetic tree generated from the ITS sequence had a greater resolution. In our ITS tree, the maximum likelihood estimation of the phylogenetic relationships divided all the samples into five groups (Fig. 3). Sheng *et al*., (2022) based on entire chloroplast data showed that *A. cochinchinensis* was more closely related to *A. racemosus* whereas Wong *et al*., (2022) chloroplast results revealed that *A. cochinchinensis* formed a sister clade to *A. officinalis* and *A. schoberioides.* According to the findings of Tian *et al*., (2023), *A. setaceus* alone was on a different branch from *A. densiflorus*, although *A. cochinchinensis* was closest to it. The phylogenetic tree by Kung & Sheng (2022) showed that *A. setaceus* formed a single group. We confirmed the position of *A. cochinchinensis* both wild and cultivated species formed a sister clade with *A. taliensis* with high support value in this study. Furthermore, two dioecious species, *A. setaceus* and *A. densiflorus,* belonged

to the same clade, with high bootstrap values. Therefore, barcoding is a potentially helpful technique for molecularly identifying the significant medicinal species of asparagus under investigation, according to our findings. There are still some discrepancies in the placement and grouping of some species (Fig. 5). To find particular links and phylogeny, more research employing improved sampling techniques and a variety of marker combinations is also required. The current study is the first in-depth investigation to evaluate the position of *A. cochinchinensis* within the genus and interrelationships among ten species of *Asparagus*. Therefore, the information generated by this study could be useful to other researchers working on the breeding, genetic enhancement, and global management of different *Asparagus* species.

Secondary structure analysis: Pertaining to the universality characteristic, extremely variable barcode loci are theoretically needed for sufficient discriminatory strength in order to provide more phylogenetic information, while adequately conservative loci are required for primer design. In actuality, there are other competitive characteristics as well, such as a long locus length to provide sufficient information against a small length for simple PCR amplification. These unjust standards have hindered the broad adoption of DNA barcoding. Because of this, a tiered approach to plant DNA barcoding has been proposed, where the first tier of resolution at the genus or family rank is a conserved DNA sequence shared by all terrestrial plants, and the second tier of resolution at the species level is a more variable region (Newmaster *et al*., 2006). In principle, the problem is resolved by this approach. But in reality, it is expensive and time-consuming to add more loci. In the secondary structure, there are several paired portions; a substitution on one side of a pair's mutation must always occur on the other for the pair to stay linked. Unlike the widely used nucleotide substitution models of today, this novel model of substitution, known as compensatory base changes, or CBCs) can incorporate extra information not present in the main sequence (Caetano-Anollés *et al*., 2002; Wolf *et al*., 2005). To enhance the collection of phylogenetic information without requiring the insertion of nucleotides, we examined alternate techniques for constructing ITS2 secondary structures in this study. According to the present study, it was shown for the first time that several key features of *A. cochinchinensis* secondary structure differed significantly from those of its closely related species (Fig. 4). These features included shape, the lacked or more stem-loop, variations in the number, size, and location of stem-loops and the size of the central ring among the four helices. Three species, *A. lycopodineus*, *A. yanyuanensis*, and *A. taliensis*, had more stem-loops in helix I and III than *A. cochinchinesis*, while the three species, *A. densiflorus*, *A. lycopodineus*, and an undiscribed species (*A. sp*2), lacked one stem-loop in helix II. The PCR sequences are unlikely to be stable and representative because each cell has numerous copies of the nrITS, which makes the application of ITS2 barcoding more difficult (Gao *et al*., 2010). The recent study found that having several copies of the ITS2 sequences is not a problem when recognizing *A. cochinchinensis* and its closely related species, highlighting the ITS2 region's universality as a DNA barcode. After comparing seven potential DNA barcodes from species of medicinal plants (*psb*A-*trn*H, *rbc*L, *mat*K, *ycf*5, *rpo*C1, and ITS/ITS2), Chen *et al*., (2010) suggested that ITS2 could be utilized as a standard DNA barcode in the future. In recent times, it has been observed that the ITS2 region primary sequences and secondary structures exhibit significant variation that is closely correlated with taxonomic classification. A number of studies have already shown how ITS2 may be used for phylogenetic reconstruction and taxonomic classification of eukaryotes; plants, animals, and fungi, at the genus and species levels (Coleman, 2007; Yao *et al*., 2010; Moorhouse-Gann *et al*., 2018). Two potential benefits of using the ITS2 for *Asparagus* species identification are that different species have different secondary metabolic products, which may influence how they are utilized in medicine, and plants in the genus *Asparagus* have unique secondary metabolic pathways (Guo *et al*., 2023). Due to their widespread international trade in medicinal and decorative *Asparagus* species, our research provides a simple means of validating these species.

Conclusions

ITS was shown to be the most appropriate DNA barcode for identifying *Asparagus* species, after evaluating all results including amplicon length, BLASTn, nucleotide composition, inter/intra-specific distance, and phylogenetic trees, were evaluated. In addition, we propose that the combination of ITS+*trn*D-*trn*F+*trn*H-*psb*A can also be effectively utilized for *Asparagus* species classification. The secondary structure of ITS2 provided another method for identifying *Asparagus* species identification. Furthermore, these results might validate these methods and aid in accurate species identification of medicinal plant, *Asparagus cochinchinensis*. This article will provide a baseline for future research in this important medicinal plant, which will serve as reference material for further studies. Authentic identification by molecular approach will facilitate modern drug discovery development. This study is not only important for Botanist but will open new avenue for chemist and biochemists to unlock the hidden compounds of these plants from this ecoregion.

Supplementary material: Supplementary A: Maximum likelihood (ML) tree. A.*trn*D-*trn*F; B. *trn*H-*psb*A; C. *trn*H-*psb*A+*trn*D-*trn*F. Bootstrap values are shown above the relevant branches.

Acknowledgement

This research is funded by the National Natural Science Foundation of China (Grant No 32350410399) to Muhammad Idrees, the Sichuan Science and Technology Program (Grant No 2022YFN0032), and Neijiang Normal University projects (Grant Nos 2020TD02, and 2022ZH02) to Hui Wang.

Author contributions

HW and MI conceived and designed the study; WL, FC, XL and QL developed the plant material; MI, ML and HW performed the DNA extraction, analyzed the data; MI, ML and MA statistical analysis; MI drafted the manuscript; HW, MA, WL, SS and ML revised the manuscript. All authors agree and approve the final version of the manuscript.

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(Received for publication 15 February 2025)