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Genotypic Variation in Agronomic Traits and Molecular Markers among Chinese Luobuma (*Apocynum* spp.) Germplasm Accessions

Yufeng Zhao [1], Runqiu Feng [1], Tao Li [1], Jahufer Mohamed Zain Zulfiqhar [2], Li Wang [3] and Jiyu Zhang [1,*]

[1] State Key Laboratory of Grassland Agro-Ecosystems, Key Laboratory of Grassland Livestock Industry Innovation, Ministry of Agriculture and Rural Affairs, College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou 730020, China; zhaoyf20@lzu.edu.cn (Y.Z.); fengrq20@lzu.edu.cn (R.F.); lit19@lzu.edu.cn (T.L.)
[2] AgResearch, Grasslands Research Centre, Private Bag 11008, Palmerston North 4442, New Zealand; zulfi.jahufer@uq.edu.au
[3] Altay GAUBAU Tea Co., Ltd., Altay 836509, China; wangli@gaubau.com
* Correspondence: zhangjy@lzu.edu.cn

Abstract: *Apocynum* spp., known as Chinese Luobuma species, are perennial herbaceous plants that not only have good ecological characteristics, such as drought resistance, salt resistance, freezing resistance, high-temperature resistance and wind sand resistance, but also have good medicinal and textile value. However, studies on the genetic variation in Chinese Luobuma are rare. In this study, the genotypic variation in the agronomic traits and molecular markers among eight germplasm accessions (referred to as genotypes) of *Apocynum* spp. was investigated. The accessions were evaluated at two locations in China, Altay and Yuzhong, during a three-year period. Analysis of the variance in yield-related traits revealed significant genotypic variation ($p < 0.05$) among the eight genotypes at the early flowering and full flowering stages. There were also significant ($p < 0.05$) genotype × year and genotype × location × year interactions for all the traits except leaf dry weight. In comparison to those evaluated at Yuzhong, the plant height, number of branches, leaf dry weight and stem dry weight at the early flowering stage were greater in Altay, with averages of 991.0 mm, 5.52, 26.41 g and 25.35 g, respectively. There were significant ($p < 0.05$) differences among genotypes in terms of the quality traits measured at the early and full flowering stages. The crude protein and crude fat content for each genotype at different locations at the early flowering stage in different years ranged from 8.64 to 10.07%. The average flavone (FLA) content was 2.31 mg/100 g. Principal component analysis (PCA) revealed that the G_1 genotype in Altay had a higher neutral detergent fiber content and leaf dry weight, and the G_2 genotype had a larger stem thickness, branch number and stem-to-leaf ratio. Five DNA sequences, *ITS*, *matK*, *psbA-trnH*, *rbcL* and *trnL-F*, were selected for analysis of the molecular variance in Chinese Luobuma. Analyses of molecular variance (AMOVA) based on the nuclear DNA sequences and chloroplast DNA sequences showed that most of the variation occurred within species. Our study indicated the significant genetic variation in Chinese Luobuma for future cultivar domestication. Genotypes with high leaf dry weights and many branches are beneficial for tea production, while tall plants with long internode lengths are valuable for the production of hemp.

Keywords: *Apocynum* spp.; agronomic traits; genetic variation; genotype-by-environment interactions; molecular markers

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1. Introduction

Chinese Luobuma is the general term for *Apocynum* spp. *Apocynum* spp. have good textile and medicinal value [1,2] and are helpful for the treatment of liver yang dizziness, palpitations, insomnia, hypertension and neurasthenia [3,4]. In recent years, due to the

deterioration of the ecological environment and excessive and aggressive mining driven by economic interests, the number of wild *Apocynum* spp. has decreased sharply, and these plants are nearly endangered [5]. In recent years, the development of related products has received a lot of attention. The leaves of *Apocynum* spp. can be used to produce tea [6] due to their medical effects, such as sleep aid and lowering blood pressure [7,8]. The stems of *Apocynum* spp. can produce hemp, which is mostly blended with other fibers [9]. In addition to *Apocynum* spp. fibers having excellent characteristics, such as moisture absorption, breathability, antistatic properties and comfort, they also reduce the frequency of far-infrared radiation [10,11]. The flowers of *Apocynum* spp. can also be used to make essential oils and produce cosmetics [12]. Studies have shown that the essential oil components in *Apocynum* spp. are mainly alcohols and esters and have inhibitory effects on *E. coli* and *Penicillium* [12]. Unfortunately, the genetic potential of these valuable plants has been underutilized due to the lack of new productive varieties with resistance to leaf rust [13,14]. The success of plant breeding depends on the extent of genetic variation in order to improve their traits. The key to detecting the genetic variation among plants, for example, in their agronomic traits, is to distinguish the genetic effects from the non-genetic effects that together make up the observed phenotype [15–17].

Agronomic traits are the result of the combined action of genes and the environment [18,19]. The phenotypic variation in agronomic traits among plants is affected not only by their growth environment but also by their individual genetics [20,21]. In *Apocynum* spp. breeding programs, increasing the biomass yield is the principal goal. A higher biomass yield improves the economic viability and sustainability of *Apocynum* spp. production [22,23]. In *Apocynum* spp., biomass was shown to be correlated with several morphological traits, such as plant height, stem diameter and branch number.

The development of DNA barcoding technology has brought new research directions to molecular biology, species classification and identification, etc. Because DNA line codes use unique DNA sequences, a large number of samples can be quickly identified using DNA barcoding through the construction of DNA libraries. At the same time, it is also a kind of molecular marker technology, which has been widely used in many research fields [24–27]. Due to their synchronous evolution, *ITS* sequences are found in many species, and there is relatively less intraspecific variation than interspecific variation [28,29]. Although the *rbcL* + *matK* composite sequence recommended by the International Plant Barcoding Working Group has a success rate of 86.3% in the identification of vascular plant taxa, there are still some taxa with a low success rate. Combined with previous studies, we believe that plant DNA barcodes should be studied in the form of multi-gene composite barcodes for specific taxa [30]. The *trnL-F* sequence has the advantages of less selection pressure and a faster evolutionary rate and is often used for phylogenetic analysis of intergeneric and subgenus taxa [31,32].

In this study, eight germplasm accessions of *Apocynum* spp. were evaluated in terms of their agronomic traits associated with yield and quality from 2017 to 2019. The objective of this study was to evaluate these accessions under field conditions to morphologically characterize them and estimate the genotypic variation among these accessions for agronomic traits. This study was conducted to assess the potential of using these eight accessions to develop base populations for future breeding programs.

2. Materials and Methods

2.1. Plant Materials and Field Trials

Trial location 1 was in Altay City in the Xinjiang Uygur Autonomous Region (E 85°31′36″–91°04′23″, N 45°00′00″–49°10′45″), and the altitude was 548 m. Based on 50 years of data recorded at the Altay weather station, the highest annual temperature is 37.6 °C, the lowest temperature is −43.5 °C, the average annual temperature is 4.5 °C, the average annual precipitation is 131–223 mm, the annual evaporation is 1367–2066 mm and the frost-free period is 123–152 days.

Trial location 2 was in Yuzhong County of Gansu Province (E 103°49′15″–104°34′40″, N 35°34′20″–36°26′30″), and the altitude was 1900 m. The annual average temperature is 6.6 °C, the annual extreme maximum temperature is 35.8 °C, the annual minimum temperature is −27.2 °C and the frost-free period lasts 100–140 days. The annual precipitation is 300–400 mm, and the evaporation is 1343.1 mm.

The eight accessions of *Apocynum* spp. are presented in Table 1 and Figure 1. A randomized complete block experimental design with 4 replicates was used at each location. Each replicate included more than 100 plants. In each 10 m × 30 m experimental plot, the plant spacing was 1 m and the row spacing was 3 m and we selected 20–50 plants from each replicate.

We collected 8 phenotype seeds, which were incubated and germinated in an incubator at 25 °C. After 15 days of germination, 10 individual plants were selected for each material, and their young tissues were collected and stored at −80 °C for DNA extraction. To ensure the quality of the samples, the A260/A280 value and concentration of the extracted genomic DNA were determined using a NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA).

Figure 1. Morphological map of eight genotypes of *Apocynum* spp.

Table 1. List of evaluated germplasm accessions.

Accession Number	Species	Phenotype
G_1	*A. venetum*	Red stems and little flowers
G_2	*A. pictum*	Red stems and medium-sized flowers
G_3		Purple spotted medium-sized flowers
G_4		Thick leaves and medium-sized flowers
G_5		Slender leaves and medium-sized flowers
G_6		Green stems and medium-sized flowers
G_7		Green stems and medium-sized flowers
G_8		Green stems and big flowers

2.2. Measurements

The agronomic traits were measured during the early flowering and full flowering growth periods during the years 2017, 2018 and 2019. The yield-related morphological traits were measured in 10 random individual plants sampled from each replicate. The traits measured were: plant height (PH/mm), stem diameter (SD/mm), number of branches (BN), internode length (IL/mm), leaf dry weight (LDW/g), stem dry weight (SDW/g) and the stem-to-leaf ratio (SLR). The leaves and stems (including inflorescences and leaf sheaths) were weighed separately to determine the stem-to-leaf ratio [33].

Quality traits: The leaves of five individual samples were mixed, crushed in a pulverizer and screened through a 1 mm sieve for further measurement; neutral detergent fiber (NDF), acid detergent fiber (ADF), crude fiber (CF), ether extract (EE), crude protein (CP) and ash were also collected. The crude fat was measured using an ANKOM XT15i automatic fat analyzer (ANKOM Technology Corporation, Beijing, China). Neutral detergent fiber, acid detergent fiber and crude fiber were measured using filter bag technology and using an ANKOM A200i semiautomatic fiber analyzer (ANKOM Technology Corporation, China). The flavone (FLA) (mg/100 g) was extracted using high-performance liquid chromatography and using an Agilent XDB C18 column in a methanol–water (65:35) mobile phase.

2.3. ANOVA

All the data collected at the Altay and Yuzhong locations were analyzed within the different growth periods for each year of the 2017, 2018 and 2019 trials. Analysis was conducted at two levels: (i) within individual locations and (ii) across years and locations.

The genotypic variation among the eight germplasm accessions for the 7 yield-related traits, 6 quality traits and flavone (FLA) was estimated by applying linear mixed-model analysis using the residual maximum likelihood (REML) [34–36] procedure in DelteGen 3.1 [37–39].

The mixed linear model:

$$Y_{ijkl} = M + g_i + l_j + (gl)_{ij} + y_k + (gy)_{ik} + (gly)_{ijk} + r_{jkl} + \varepsilon_{ijkl}$$

where Y_{ijkl} is the value of an attribute measured from accession i in replicate l in location j in year k and $i = 1..., n_g$, $j = 1..., n_l$, $k = 1..., n$ and m is the mean value; g_i is the random genotypic effect of accession i, $N(0, \sigma^2_g)$; l_j is the fixed effect of location j; y_k is the fixed effect of year k; $r_{jk}l$ is the random effect of replicate l within location j, in year k, $N(0, \sigma^2_b)$; $(gl)_{ij}$ is the effect of the interaction between accession i and location j, $N(0, \sigma^2_{gl})$; $(gy)_{ik}$ is the effect of the interaction between accession i and year k, $N(0, \sigma^2_{gy})$; $(gly)_{ijk}$ is the effect of the interaction between accession i, location j and year k, $N(0, \sigma^2_{gly})$ and ε_{ijkp} is the residual effect for accession i in replicate l in location j and year k, $N(0, \sigma^2_\varepsilon)$.

2.4. Pattern Analysis

Pattern analysis, a combination of cluster analysis and principal component analysis, was conducted to provide a multi-trait graphical summary of the performance of the eight germplasm accessions. This analysis was based on the accession-by-trait BLUP matrix constructed using the individual trait outputs generated from the REML analysis across years. Only traits with significant ($p < 0.05$) genotypic variation among the eight accessions were included in the analysis, which was conducted using the DeltaGen 2.0 software.

2.5. Phenotypic Correlation

The phenotypic correlation coefficients among the eight accessions for the traits measured were estimated using the multivariate analysis option in DelteGen.

2.6. Genetic Variation in Molecular Markers

ITS, *psbA-trnH*, *matK*, *trnL-F* and *rbcL* were selected for PCR amplification. The sequences of primers used are listed in Table S7. The PCR mixture included 12.5 μL of 2× Master Mix, 2.5 μL of the upstream and downstream primers, 2.5 μL of 50 ng/μL template DNA and 5 μL of dd H_2O. The PCR procedure was as follows: pre-denaturation at 95 °C for 3 min; 35 cycles of denaturation at 94 °C for 30 s; annealing at 55 °C for 40 s; extension at 72 °C for 50 s; extension at 72 °C for 7 min and preservation at 4 °C. The PCR amplification products were detected using 1% agarose gel electrophoresis, and products of the appropriate fragment sizes and meeting the sequencing standards were subsequently sent to Sangong Bioengineering (Shanghai, China) Co., Ltd., for Sanger sequencing. The Chromas software (version 2.22) and the Sequencher software (version 4.8) were used for sequence correction and manual calibration of the DNA sequencing results. The MEGA 7.0 software was used for sequence alignment, and neighbor-joining (NJ) was used to construct a phylogenetic tree for the clustering. AMOVA was performed using Arlequin version 3.5 [40].

3. Results

3.1. Genotypic Variance Components of Yield-Related Traits

The analysis of variance indicated significant ($p < 0.05$) genotypic variance in the yield-related traits at the early flowering stages across years 2018 and 2019 and across locations in Altay and Yuzhong (Table 2). The accession-by-year interaction effects were significant ($p < 0.05$) for the traits PH, SD, LDW and SDW. There were also significant ($p < 0.05$) accession-by-year-by-location interactions for all the traits except for the stem dry weight and leaf dry weight. There was no significant difference ($p > 0.05$) in the annual genotypic variation. The mean plant height across years ranged from 66.41 cm to 77.98 cm, and the mean stem diameter and internode length were 0.43 cm and 3.73 cm, respectively. The leaf dry weight ranged from 19.15 g to 21.77 g.

Analysis of the data collected from Altay showed significant ($p < 0.05$) genotypic variance among the eight accessions and an interaction between the accession and year for yield-related traits at the early flowering stage from 2017 to 2019 (Table S1). There was significant ($p < 0.05$) genotypic variance among the accessions for the yield-related traits and the accession-by-year interactions in Yuzhong at the early flowering stage from 2017 to 2019 (Table S2).

The analysis of variance indicated significant ($p < 0.05$) genotypic variance among the eight accessions for the yield-related traits at the full flowering stages from 2017 to 2018 in Altay, except for the trait of IL (Table 3). There were also significant ($p < 0.05$) genotype-by-year interactions, except for with SLR. However, there was no significant ($p > 0.05$) variation among years, except for with SD. The mean plant height among the accessions in different years ranged from 54.34 cm to 82.77 cm. The mean stem diameter and internode length were 0.39 cm and 4.03 cm, respectively, and the leaf dry weight ranged from 10.19 g to 16.45 g.

Table 2. Mean, range, least significant difference ($LSD_{0.05}$), genotypic effect (σ^2_g), year effect (σ^2_y), genotype-by-year interaction (σ^2_{gy}), genotype-by-location interaction (σ^2_{gl}), genotype-by-year-by-location interaction (σ^2_{gly}), experimental error (σ^2_ε), variance components and associated standard errors (±SE), estimated from across-year and across-location analyses among the eight Chinese Luobuma germplasm accessions for yield-related traits measured during the early flowering stage in the years 2018 and 2019 in Altay and Yuzhong.

	PH (mm)	SD (mm)	IL (mm)	BN	LDW (g)	SDW (g)	SLR
Mean	714.5	4.3	37.3	4.39	20.13	18.45	0.86
Range	664.1–779.8	3.9–4.5	34.5–40.9	3.75–4.88	19.15–21.77	17.52–19.09	0.79–0.96
$LSD_{0.05}$	88.11	0.26	1.41	11.03	20.12	13.77	0.86
σ^2_g	841.23 ± 95.56	5.57 ± 0.36	1.87 ± 0.28	3.63 ± 0.62	29.07 ± 14.48	0.07 ± 0.001	0.04 ± 0.005
σ^2_y	ns	ns	ns	ns	ns	ns	ns
σ^2_{gy}	29.05 ± 12.73	0.06 ± 0.03	ns	ns	17.21 ± 8.49	21.97 ± 7.94	ns
σ^2_{gl}	ns	ns	ns	ns	ns	ns	ns
σ^2_{gly}	46.02 ± 19.50	0.09 ± 0.04	0.22 ± 0.07	0.42 ± 0.20	ns	ns	0.0009 ± 0.0003
σ^2_ε	591.57 ± 33.99	2.92 ± 0.17	8.09 ± 0.45	9.78 ± 0.54	79.28 ± 5.31	77.21 ± 6.22	0.02 ± 0.002

PH, plant height; SD, stem diameter; IL, internode length; BN, branch number; LDW, leaf dry weight; SDW, stem dry weight; SLR, stem-to-leaf ratio; ns, not significant.

Table 3. The trait mean, range, least significant difference ($LSD_{0.05}$), genotypic effect (σ^2_g), year effect (σ^2_y), genotype-by-year interaction (σ^2_{gy}) and experimental error (σ^2_ε) variance components and associated standard errors (±SE) were estimated for eight Chinese Luobuma accessions for yield-related traits measured at the full flowering stage from 2018 to 2019 at the Altay location.

	PH (mm)	SD (mm)	IL (mm)	BN	LDW (g)	SDW (g)	SLR
Mean	685.6	3.9	40.3	5.08	14.90	15.37	0.90
Range	543.4–827.7	3.3–4.8	33.4–43.4	3.98–5.99	10.19–16.45	10.00–17.63	0.74–1.06
$LSD_{0.05}$	88.95	0.21	1.08	10.32	26.83	70.81	0.66
σ^2_g	890.40 ± 77.45	0.003 ± 0.001	ns	11.71 ± 1.44	80.25 ± 8.17	574.91 ± 27.31	0.04 ± 0.008
σ^2_y	ns	0.04 ± 0.02	ns	ns	ns	ns	ns
σ^2_{gy}	44.70 ± 13.86	0.003 ± 0.0008	0.23 ± 0.07	0.92 ± 0.38	5.17 ± 1.73	8.97 ± 2.79	ns
σ^2_ε	121.03 ± 7.18	0.01 ± 0.0006	0.75 ± 0.04	10.60 ± 0.62	12.68 ± 0.88	16.58 ± 1.15	0.16 ± 0.01

PH, plant height; SD, stem diameter; IL, internode length; BN, branch number; LDW, leaf dry weight; SDW, stem dry weight; SLR, stem-to-leaf ratio; ns, not significant.

3.2. Genotypic Variance Components for Nutritional Quality Traits

The analysis of variance indicated significant ($p < 0.05$) genotypic variation in the nutritional quality traits among the eight accessions at the early flowering stage of 2018 across the two locations, Altay and Yuzhong (Table 4). These trait means and ranges indicated wide phenotypic variation in the nutritional quality traits and FLA content in the *Apocynum* spp. The genotypic variances estimated among the eight accessions for all the different traits measured were significant ($p < 0.05$) (Table 4). There were no significant ($p > 0.05$) differences among locations and no genotype-by-location interaction variance, except for in CP and FLA. The crude protein content in different years ranged from 12.18% to 16.77%, the average crude fiber and ash contents were 22.16% and 11.28%, respectively, and the average FLA content was 2.12 mg/100 g.

In Altay, there was significant ($p < 0.05$) genotypic variance among the eight accessions in the nutritional quality traits during the early flowering stages, from 2017 to 2018, except for the traits CP and NDF (Table S3). There were no significant ($p > 0.05$) differences among years or in the genotype-by-year interaction variance, except for that of CP. Compared to those in the full flowering stage, the early flowering stage presented higher levels of NDF, ADF and FLA (33.97%, 79.09% and 2.64 mg/100 g, respectively). There was significant ($p < 0.05$) genotypic variance in the nutritional quality traits among the eight accessions at the full flowering stage during 2017 and 2018 (Table S4). There were no significant ($p > 0.05$) differences among the years or among the genotype-by-year interactions, except for in the

CP and FLA. In Yuzhong, there was significant ($p < 0.05$) genotypic variance among the eight accessions for the nutritional quality traits during the early flowering stage in 2018, except for in the CP, NDF and CF (Table S5).

Table 4. The nutritional quality trait mean, range, least significant difference ($LSD_{0.05}$), genotypic effect (σ^2_g), location effect (σ^2_l), genotype-by-location interaction (σ^2_{gl}), and experimental error (σ^2_ε) variance components and associated standard errors (±SE) were estimated among eight Chinese Luobuma accessions evaluated at the early flowering stage from 2018 to 2019 in Altay and Yuzhong.

	CP (%)	NDF (%)	ADF (%)	EE (%)	CF (%)	Ash (%)	FLA (mg/100 g)
Mean	15.36	26.83	69.14	7.48	22.16	11.28	2.12
Range	12.18–16.77	22.88–30.39	66.05–73.81	4.88–13.54	18.68–24.73	9.73–12.09	1.94–2.36
$LSD_{0.05}$	18.32	9.09	21.21	40.08	7.71	16.18	0.73
σ^2_g	37.79 ± 3.26	8.05 ± 2.46	44.19 ± 11.32	174.07 ± 30.56	0.02 ± 0.02	29.57 ± 2.45	0.03 ± 0.01
σ^2_l	ns	ns	ns	ns	ns	ns	ns
σ^2_{gl}	1.48 ± 0.69	ns	ns	ns	ns	ns	0.05 ± 0.02
σ^2_ε	2.01 ± 0.42	12.07 ± 2.13	43.62 ± 7.58	87.69 ± 15.03	45.29 ± 8.16	1.71 ± 0.37	0.05 ± 0.01

CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ether extract; CF, crude fiber; FLA, flavone; ns, not significant.

3.3. Pattern Analysis

The biplot generated from the PCA based on the yield and quality traits measured in Altay during the full flowering stages in 2017 and 2018 indicated that the eight germplasm accessions were clustered into three groups (Figure 2). In group 2, accessions G_2, G_3, G_4 and G_6 had a greater average stem diameter, stem-to-leaf ratio, number of branches and amount of crude protein. Accession G_1 had high neutral detergent fiber.

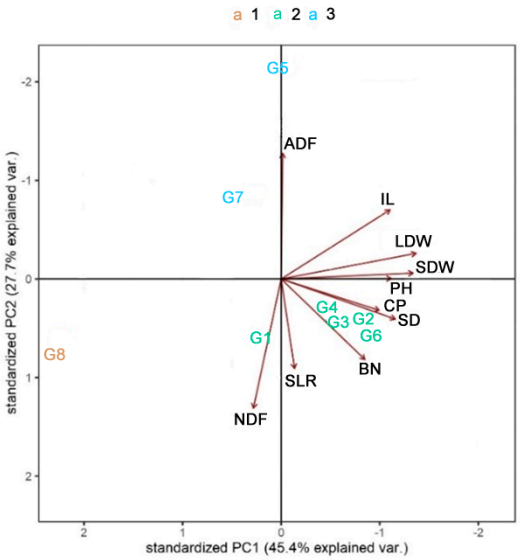

Figure 2. Principal component analysis of agronomic traits of different genotypes of *Apocynum* spp. and *Poacynum* spp. during the full flowering stage from 2018 to 2019 in Altay. Different colors represent different groups. PH, plant height; SD, stem diameter; IL, internode length; BN, branch number; LDW, leaf dry weight; SDW, stem dry weight; SLR, stem−-to−leaf ratio; ns, not significant; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; Different colored a represents genotypes with the same trend.

According to the biplot generated from the PCA using the yield and quality traits measured in Altay during the early flowering stages in 2017 and 2018 (Figure S2), the first principal component explained 34.8% of the total trait variation. The above-average plant height, branch number, internode length and leaf dry weight were shown for accessions

G_3, G_4 and G_5 in group 4. Accession G_1 had a high crude fiber content and stem diameter. Accession G_7 had a high stem-to-leaf ratio. A biplot (Figure 3) generated from the PCA of the yield and quality traits measured in Yuzhong during the early flowering stages in 2018 and 2019 indicated six accession groups. Accessions G_2, G_3 and G_4 had above-average stem diameters and acid detergent fiber contents. Accession G_1 had high amounts of neutral washing fibers and crude fat and a high internode length, crude fiber content and branch number.

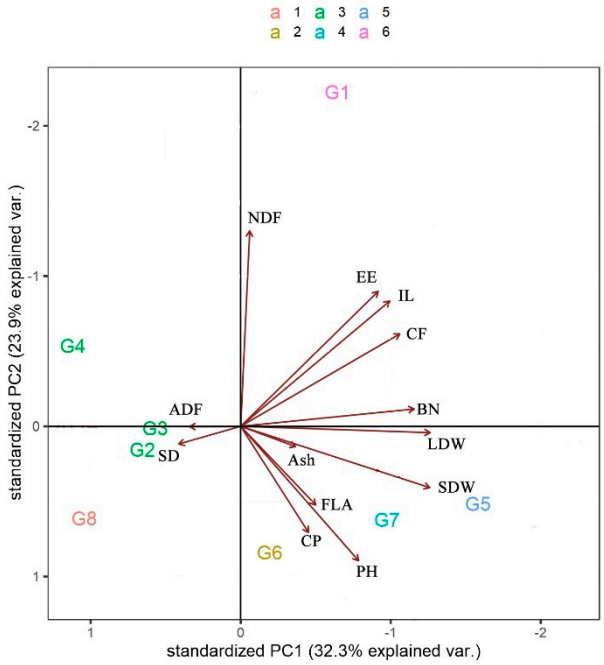

Figure 3. Principal component analysis of agronomic traits of different genotypes of *Apocynum* spp. and *Poacynum* spp. during the early flowering stage from 2018 to 2019 in Yuzhong. Different colors represent different groups. PH, plant height; SD, stem diameter; IL, internode length; BN, branch number; LDW, leaf dry weight; SDW, stem dry weight CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ether extract; CF, crude fiber; FLA, flavone. Different colored a represents genotypes with the same trend.

3.4. Phenotypic Correlation

The phenotypic correlation coefficients among the eight accessions for the traits measured during the early flowering stage in Altay are presented in Table 5. The correlation coefficients ranged from strongly to weakly positive or negative pairwise associations between the 12 traits. Of special interest are the phenotypic correlations between the FLA and the other traits. The correlation coefficients between the FLA and PH and between the FLA and IL were −0.86 and −0.71, respectively, indicating a strong negative phenotypic correlation ($p < 0.05$).

The phenotypic correlation coefficients among the accessions for the different traits measured during the early flowering stage in Yuzhong are presented in Table S6. These coefficients ranged from strongly to weakly positive or negative pairwise associations between the 14 traits. The correlation coefficients between LDW and SDW and between LDW and BN were 0.93 and 0.63, respectively, indicating significant positive phenotypic correlations (Table S6).

Table 5. Phenotypic correlation coefficients among the eight germplasm accessions of Chinese Luobuma based on trait means across the early and full flowering stages and across different years in Altay.

Trait	SD	IL	BN	LDW	CP	NDF	ADF	EE	CF	FLA	Ash
PH	0.23	0.56	0.66 *	0.40	−0.50	0.03	−0.14	0.39	−0.51	−0.86 **	0.39
SD		0.38	−0.41	0.63	−0.20	−0.10	0.08	−0.17	0.13	−0.47	0.18
IL			0.26	0.39	−0.51	−0.64	−0.13	0.31	−0.72 *	−0.71 *	0.53
BN				0.08	−0.48	−0.09	−0.44	0.03	−0.38	−0.53	0.27
LDW					0.68 *	0.18	0.33	−0.17	−0.23	−0.39	0.63
CP						0.19	−0.15	0.20	0.31	0.47	−0.93 **
NDF							−0.53	0.18	0.16	0.37	−0.18
ADF								0.38	−0.22	0.46	0.41
EE									−0.72	−0.08	0.00
CF										0.33	−0.42
FLA											−0.31

*, ** indicate significance at the 0.05 and 0.01 probability levels, respectively. PH, plant height; SD, stem diameter; IL, internode length; BN, branch number; LDW, leaf dry weight; ns, not significant; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ether extract; CF, crude fiber; FLA, flavone.

3.5. Genotypic Variance Components of Molecular Markers

The PCR amplification efficiency and sequencing success rate are important indices for evaluating molecular markers. The analysis of each DNA sequence showed that the percentage range of GC content was 34.78–61.05%. The GC content was highest in the *ITS* sequence and lowest in the *matK* sequence. The number of variation sites ranged from 5 to 37, among which the *psbA-trnH* sequence had the most variation sites (Table S8).

The analyses of molecular variance (AMOVAs) for Chinese Luobuma based on the *ITS* sequences revealed that most of the variation occurred within groups. For the combined sequences of *matK+psbA-trnH+trnL-F+rbcL*, most of the variation also occurred within groups (Table 6). Cluster analysis of eight genotypes based on five sequences was performed using neighbor-joining, and a dendrogram was inferred (Figure 4). The sequence comparison of *ITS*, *mat*K, *psb*A-*trn*H, *rbc*L and *trn*L-F in eight genotypes and the gel diagram showed that the sequence differences of five molecular markers could be clearly displayed (Figures S3 and S4). *ITS*, *matK*, *rbcL* and *trnL-F* can be used to divide all accessions into two major clusters and distinguish G1 from the other genotypes. The composite sequence *matK+psbA-trnH+trnL-F+rbcL* can also be used to divide all the genotypes into two clusters. However, we found that G1 and G3 were clustered together (Figure S5). The groups were generated according to species type; most *Apocynum* spp. accessions were grouped together, as was G8 (Figure 4). *psbA-trnH* could not distinguish the eight genotypes because the sequence was short and the similarity was high. We found that not all single-molecule marker techniques were able to distinguish the eight genotypes at the species level.

Table 6. Analyses of molecular variance (AMOVAs) for Chinese Luobuma based on five DNA sequences.

Sequence	Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation	F_{st}
Nuclear sequence *ITS*	Among groups	1	0.45	0.12	33.33	0.33
	Within groups	6	1.43	0.24	66.67	
Chloroplast DNA sequences *(matK+psbA-trnH+rbcL+trnlL)*	Among groups	1	7.13	1.24	26.57	0.27
	Within groups	6	20.5	3.42	73.43	

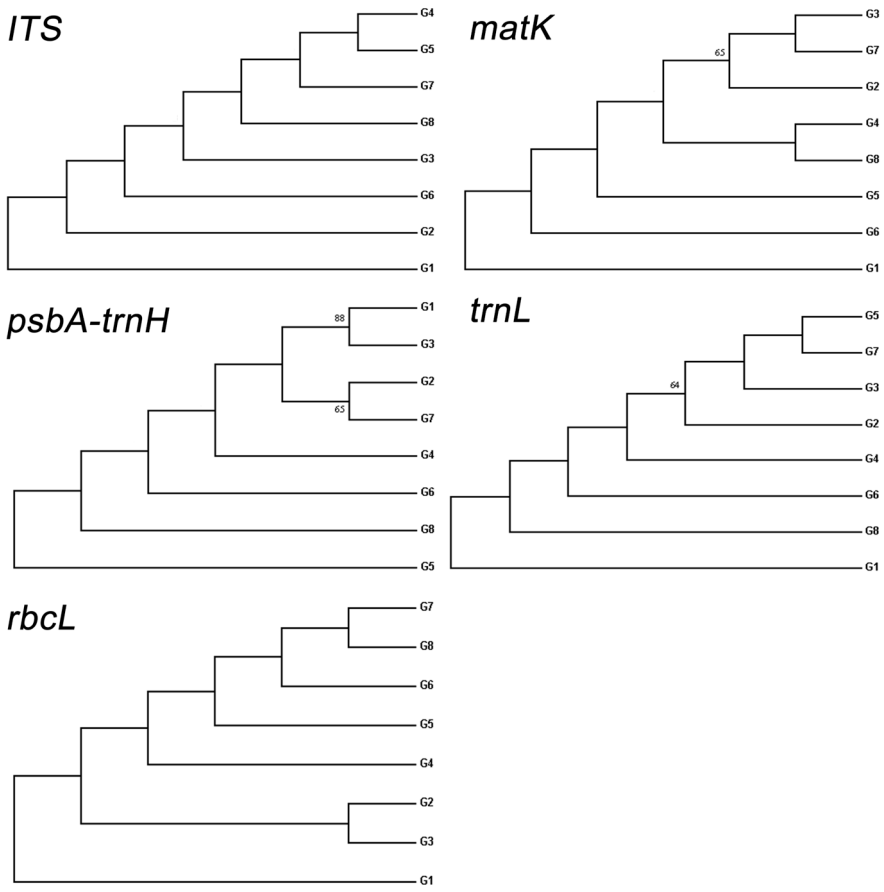

Figure 4. Neighbor-joining (NJ) tree for different genotypes based on five DNA sequences, *ITS*, *matK*, *psbA-trnH*, *rbcL* and *trnL-F*.

4. Discussion

A. venetum has a good ecological restoration ability and good feeding value. Moreover, these plants are taller and contain more branches, which can aid in wind prevention and sand fixation. This approach is helpful for ecological restoration [41]. There is good evidence that plant breeding has successfully improved populations whenever there is genetic variation within germplasm pools, and selection has been focused on the right traits being measured in the appropriate environments [42]. Previous studies have reported the results of germplasm evaluation in terms of pest and disease resistance in *Apocynum* spp. [43,44] and have economic value for agriculture, medicine and industry [25]. However, very few varieties are registered in China. The development of new varieties of *Apocynum* spp. has become a priority in China to increase the utilization of these valuable species [25].

Information on the phenotypic and genotypic diversity in germplasms in terms of the agronomic traits associated with breeding objectives enhances the development of appropriate breeding methods. Estimates of the genotypic and genetic variation in agronomic traits have been reported for the *Gossypium barbadense* [25], *Glycine max* [45], *Melilotus officinalis* [46] and *Oryza sativa* subspecies [47] and many other plant species [48–50].

In our study, analysis of the agronomic traits showed that *Apocynum* spp. are tall plants with a high number of branches and high leaf yield. These characteristics make these species useful for ecological restoration [51]. The stems of *Apocynum* spp. can produce hemp, and the internode length is a direct indicator of the length and toughness of the hemp plants. Therefore, the internode length measured in our study can provide a basis for selecting and breeding specific varieties of industry hemp [52,53]. *Apocynum* spp. show a good forage palatability with a high leaf dry weight and low crude fiber and crude ash contents [54,55]. Tea prepared from *Apocynum* spp. leaves has gained popularity as a

nutritional supplement beverage for anti-aging purposes [56]. The flavonoid content in Luobuma tea is an important indicator of the tea quality [57], as flavonoids can scavenge free radicals [58,59]. Therefore, to select high-quality *Apocynum* spp. plants, we measured the flavonoid content. We found that the flavonoid content was 2.12 mg/100 g during the early flowering stage. Moreover, the flavonoid content during the full flowering stage was greater than that during the early flowering stage in the same year and at the same location.

The presence of genotype × environment interactions complicates the selection of material for broad adaptation due to variable relative performances across environments [55]. Quantifying the magnitude and understanding the causes of genotype × environment interactions can be helpful when planning breeding strategies [60,61]. Studies have reported that a range of traits in white clover, especially yield-related traits, are sensitive to genotype × environment interactions [62–64]. In our study, most of the traits exhibited significant differences under interactions between genotype and environment, indicating the importance of multisite evaluation. In this study, the yield and quality traits of *Apocynum* spp. were evaluated in Altay and Yuzhong, China. The results showed that there were significant genotypic differences in the yield traits among the genotypes and among the genotype × year and genotype × year × location interactions ($p < 0.05$). There were significant differences in the quality traits among the genotypes ($p < 0.05$).

The application of pattern analysis in this study provided a graphical summary of the yield and quality traits of the *Apocynum* spp. evaluated in different years and locations. These results will aid in the identification of genotypes with trait combinations beneficial for developing varieties for tea and hemp production. The principal component analysis (PCA) revealed that G1 had a greater leaf dry weight, branch number and plant height, while G2 had a greater stem diameter, internode length and plant height. In a similar study, Luo et al. (2018) [22] used pattern analysis to examine the associations among key agronomic traits of *Melilotus albus* and identified material for breeding new varieties with a high yield and a low coumarin content. Correlation analysis revealed that the crude protein content was positively associated with leaf dry weight, while the flavone content was negatively correlated with the plant height and internode length. These relationships between traits provide a basis for the future selection of new *Apocynum* spp. varieties (lines) with favorable yield and quality traits. From the different genotypes of *Apocynum* spp., we screened out the genotypes conducive to tea production, with a large leaf dry weight and more branches, and the genotypes conducive to hemp production, with tall plants, long internodes and large stems, which can a provide theoretical basis for breeding new varieties (lines) of *Apocynum* spp. with good agronomy and quality traits and suitable for domestic popularization.

The genetic background of *Apocynum* spp. is complex, and there are many genotypes affected by habitat changes. *Apocynum* spp. varieties are cultivated through genetic selection in different environments. In this study, cluster analysis of the eight genotypes of *Apocynum* spp. based on different barcodes found that there was a large genetic difference between G4 and the other genotypes, suggesting that there might be gene exchange between G4 and other genotypes, or it might be a heterozygote with multiple parental sources. Molecular marker technology is based on the nucleotide sequence variation in genetic material between individuals [23,65]. It is often used to detect differences between organisms. Compared with morphological, biochemical and cytological markers, molecular markers have many advantages [66]. For example, most molecular markers are codominant, and it is very convenient to select recessive traits [67,68]. The success rate of individual DNA barcodes for plant identification varies, especially in hybrids or varieties with a gene penetration phenomenon [30]. Scholars have commonly chosen DNA barcodes from nucleotide gene sources and chloroplast sources for plant identification and found that the DNA barcodes from the nuclear gene source had higher species-specific differences [69]. Of course, our results are similar to those of previous studies. On the other hand, DNA barcodes evolve at different rates from chloroplast-derived DNA barcodes, and the identification success rate is also different [23]. This study also confirmed that the

chloroplast-derived DNA barcodes in different genotypes of *Apocynum* spp. had fewer loci of variation (except *psb*A-*trn*H, the barcode with the most loci of variation), and the genetic distance of each barcode was less different among different genotypes.

The success rate of individual DNA barcodes for plant identification varies, especially in hybrids or varieties with gene penetration phenomena [30]. Therefore, some scholars have proposed using DNA barcode sequence combination to solve the problem. The *arp*F-*atp*H+*psb*K-*psb*L+*trn*H-*psb*A combination barcode was used to identify Orchidaceae plants, and the identification success rate was 98.8% [23]. In this study, phylogenetic tree analysis of different barcodes showed that some individual barcodes were less able to distinguish different genotypes of *Apocynum* spp., and the eight genotypes could not be distinguished at the genetic level using different combinations of barcodes. Genomes are rich in variation, and the number of molecular markers is almost unlimited []. In our study, the analyses of molecular variance (AMOVAs) for Chinese Luobuma based on the nuclear and chloroplast sequences showed that most of the variation occurred within species. Using their agronomic traits and DNA barcoding technology, phenotypic morphological analysis and studies on the molecular genetic variation in different genotypes of *Apocynum* spp. were conducted, aiming to reveal the phenotypic differences in different genotypes of *Apocynum* spp. and provide a theoretical basis for the breeding of new varieties (lines) with a high yield and quality.

5. Conclusions

The significant genotype × year and genotype × year × location interactions estimated for the yield traits across the two locations, Yuzhong and Altay, indicate the importance of conducting multilocation trials to develop new broadly adapted varieties in China. The estimates of the genotypic variation indicated the potential genetic variation available in the key agronomic traits of *Apocynum* spp. At the Altay site, there were significant differences between the genotypes in the quality traits at the early flowering stage and the full flowering stage ($p < 0.05$). Principal component analysis found that the genotype G1 in Altay has a higher neutral detergent fiber content and leaf dry weight, and the genotype G2 has a larger stem thickness, branch number and stem-to-leaf ratio.

The barcodes *mat*K, *rbc*L and *trn*L-F could divide all genotypes into two groups, which can distinguish *Apocynum* spp. and G8. *ITS*, *matK*, *rbcL* and *trnL-F* can be used to divide all the genotypes into two major clusters. The analyses of molecular variance (AMOVAs) for Chinese Luobuma based on five sequences showed that most of the genetic variation occurred within species. For the *matK*, *psbA-trnH*, *trnL-F* and *rbcL* sequences, most of the variation presented among the genotypes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14030332/s1, Figure S1: Distribution map of sampling locations. Figure S2: Principal component analysis of agronomic traits in different genotypes of *Apocynum* spp. and *Poacynum* spp. during early flowering stage from 2017-2019 at Altay. Figure S3: gel pictures of five molecular markers for 8 genotypes. Figure S4: Comparison of sequences for eight genotypes ITS, matK, psbA-trnH, rbcL, trnL-F. Figure S5: Neighbor-joining (NJ) tree for different phenotypes using matK + psbA-trnH + rbcL + trnL markers. Table S1: Trait mean, range, least significant differences (LSD0.05), genotypic (σ^2_g), year (σ^2_y), accession-by-year interaction (σ^2_{gy}), and experimental error (σ^2_ε) variance components, and associated standard errors (±SE), estimated from 8 accessions, for yield related traits at the early flowering stage from 2017 to 2019 at Altay. Table S2: Trait average, range, least significant differences (LSD0.05), genotypic (σ^2_g), year (σ^2_y), genotype × year interaction (σ^2_{gy}), and experimental error (σ^2_ε) variance components, and associated standard errors (±SE), estimated from 8 genotypes, evaluated yield related traits at early flowering stage from 2018 to 2019 at Yuzhong. Table S3: Nutritional quality average, range, least signifcant differences (LSD0.05), genotypic (σ^2_g), year (σ^2_y) and genotype with year (σ^2_{gy}), and experimental error (σ^2_ε) variance components, and associated standard errors (±SE), estimated from 8 genotypes, evaluated in early flowering stage at Altay. Table S4: Nutritional quality average, range, least significant differences (LSD0.05), genotypic (σ^2_g), year (σ^2_y) and genotype with year (σ^2_{gy}), and

experimental error (σ^2_ε) variance components, and associated standard errors (±SE), estimated from 8 genotypes, evaluated in full flowering stage at Altay. Table S5: Nutritional quality average, range, least significant differences (LSD0.05), genotypic (σ^2_g), and experimental error (σ^2_ε) variance components, and associated standard errors (±SE), estimated from 8 genotypes, evaluated in early flowering stage at Yuzhong. Table S6: Correlation analysis of characters of Apocynum and Poacynum of different genotypes during early flowering stage from 2017 to 2018 at Yuzhong. Table S7: Primer information of different gene segments. Table S8: Sequences information of five sequences.

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