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# Genetic diversity and population structure analysis for *Ampelopsis grossedentata* using microsatellite markers

**Xiao-ying LI[1], Lin WEI[1,2,*]**

[1]College of Biology and Food Engineering, Huaihua University, Huaihua, China.
[2]College of Basic Medicine, Guizhou University of Traditional Chinese Medicine, Guiyang, China

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**Abstract:** *Ampelopsis grossedentata* is an important national medicine in China. It is mainly used commonly by the Yao, Miao, Dong, and other ethnic minorities of China. The current literature, however, contains no study on the molecular genetics and taxonomy of *A. grossedentata*, which had led to potential problems in the conservation and utilization of this resource. Therefore, the present study was aimed to use simple sequence repeat (SSR) markers for assessing the levels and the distribution of genetic diversity in a sample set of 225 individuals from nine populations of *A. grossedentata* in China. The genetic differentiation among the nine populations was estimated at the level of 27 microsatellite loci. All loci were polymorphic in the nine populations analyzed. The average value of the Wright's fixation index (*Fis*) was –0.1154, with the range of –0.4627 to 0.2399, at the population level. The average *Fit* value was –0.0119, with the range of –0.3523 to 0.3563, at the population level. The *Fst* value was 0.0928 on average, ranging from 0.0463 to 0.1567. The SSR analysis revealed high levels of genetic variations within the studied populations, while the genetic differentiation among the populations was low. This finding probably reflected the historical association among the different populations of *A. grossedentata*. It is possible that sexual reproduction in the natural breeding system of *A. grossedentata* increased the levels of genetic variation within the populations while it reduced the genetic differences among the populations. The genetic cluster analysis revealed that the basal clade comprised populations from South China and that the different populations of *A. grossedentata* were categorized into five branches, which had continuous southeastward and southwestward distributions. The genetic structure of the populations and the levels of genetic diversity of *A. grossedentata* observed in the present study could provide important insights into the current status and the protection value of this species.

**Key words:** *Ampelopsis grossedentata*, genetic diversity, population structure, microsatellite markers

## 1. Introduction

*Ampelopsis grossedentata* (hand.-Mazz.) W.T. Wang is an important national medicine in China. It is mainly used commonly by the Yao, Miao, Dong, and other ethnic minorities of China (Editorial board of Chinese materia medica of State Administration of traditional Chinese medicine, 1999). The stem, leaf, and root of *A. grossedentata* plant are used for treating cold, fever, sore throat, jaundice, and hepatitis. According to Yao medicine claims, *Ampelopsis grossedentata* is also capable of treating heatstroke, dizziness, and upset (Xie et al., 1975; Zhao et al., 2009). In Dong medicine, the whole plant of *Ampelopsis grossedentata* is used for treating sores (Xie et al., 1975;Zou, 2011). The 'Chinese Materia Medica' documents that *A. grossedentata* has the effect of clearing away heat and detoxification, diuresis, and detumescence (Editorial board of Chinese materia medica of State Administration of traditional Chinese medicine, 1999). *Ampelopsis grossedentata* is mainly used for treating cold and fever, sore throat, red-eye swelling and pain, huanghuan hepatitis, acute conjunctivitis, carbuncle, swelling, and furuncle. Shanxi Bailu Pharmaceutical Co. Ltd. used *A. grossedentata* as the raw material to produce a drug that was named *Ampelopsis grossedentata* total flavonoids buccal tablets (approval number: z20090046). This drug is mainly used for detumescence, pain relief, detoxification, and wound healing. *A. grossedentata* is sweet, light, and cool in flavor. The young stems and leaves of the plants of this species have been used traditionally for preparing a 'health tea', commonly referred to as the 'rattan tea', which has been used for hundreds of years in China. The earliest records on Rattan tea are in the ancient books, including Yin Shan Zheng Yao, Cao Mu Bian Fang, Peng folk rhyme, Guo bang, etc. In modern times, *A. grossedentata* has become an important raw material in national medicine. Nowadays, *A. grossedentata* is known by different names

* Correspondence: weilin187@gzy.edu.cn

in different regions and nationalities based on the different harvest times, processing methods, and national cultures. Recent studies have also reported several medicinal effects, such as the antiinflammatory (Hou et al., 2015; Chen et al., 2018; Renata et al., 2020), antibacterial (Zhang et al., 2009; Fan et al., 2018; Xia et al., 2019), antitumor, antiviral (Zhou et al.,2020), and antioxidant effects (Zhang et al., 2003; Gao et al., 2009; Chen, 2011; Liao et al., 2014; Ye et al., 2015; Xie et al., 2019; Zhang et al., 2019; Jia et al., 2021), melanogenesis inhibition ( Huang et al., 2016), lowering of blood glucose (Zhong et al., 2002; Chen et al., 2016; Ran et al., 2019; Li et al., 2021; Li et al., 2022) and blood lipid levels (Zhong et al., 2002), lowering of blood pressure (Huang et al., 2018), and liver protection effects (Fan et al., 2020; Li et al., 2021; Li et al., 2022) of the national medicinal material *A. grossedentata*. Therefore, *A. grossedentata* is used widely and also has great development prospects.

Rapid developments in the field of molecular biology have enabled overcoming the limitations of the phenotypic evaluation of germplasm and provided important tools for the effective and sustainable utilization of *A. grossedentata* as an important medicinal resource. Among the best molecular markers are the simple sequence repeats (SSRs), which are distributed widely in the genome of eukaryotic cells (Bell and Ecker, 1994; Vieira et al., 2016). The principle underlying the SSR biomarker approach is to design site-specific primers based on the sequences of the two flanking regions of microsatellite DNA and then amplify a single microsatellite site using PCR (Tautz D, 1989; Bell and Ecker, 1994). Subsequently, based on the length of the amplified products, the polymorphism of the different genotypes in the SSR sites is determined. The SSR approach presents the advantages of codominance, good repeatability, abundant polymorphism, and simple experimental procedure (Elibol and Bilgen, 2017; Evren and Kaya, 2021). Therefore, this technique has great potential in the analysis of genetic diversity and population structure differentiation. However, the current literature contains no report on the use of this technique in determining the genetic diversity of *A. grossedentata*.

In the present study, the genetic diversity and population structure of *A. grossedentata* were analyzed based on SSR molecular markers in a large number of individual plants sampled from a wide range of areas, including the south, southeast, central south, southwest, and west regions of China. The main objectives were to determine the levels of genetic diversity and differentiation among the populations within the species and to analyze the genetic structure differentiation and the evolutionary history of *A. grossedentata*. In addition, based on the revealed genetic information, the strategies for the development, effective utilization, and resource conservation in regard to *A. grossedentata* were proposed.

## 2. Methods and experiments

### 2.1. Sample collection

Leaf samples were collected from 225 individuals of *A. grossedentata*. Only the plants that were at a minimum distance of 500 m from each other within each population were sampled to prevent sampling clonal relatives. The selected samples were from nine different populations located in the natural distribution of *A. grossedentata* in the nine provinces of China, including the Hunan province, Guizhou province, and Yunnan province. Further details are provided in Table 1 and Figure 1. The leaf samples were immediately dried and preserved in silica gel until use for DNA extraction.

### 2.2. DNA extraction and primer screening

The genomic DNA was extracted from the leaves of each sample plant using the improved CTAB protocol (Doyle, 1991). The extracted genomic DNA was dissolved in deionized double distilled water and then quantified using a spectrophotometer. The observed band intensities of the samples on 18% polyacrylamide gels were compared with those of the known standards of lambda DNA. In a preliminary experiment, 50 pairs of SSR primers selected from the transcriptomic data of *A. grossedentata* available in the laboratory were tested on nine individuals selected randomly from all the populations included in the present study. Among the tested primers, the 27 pairs of primers that produced distinct bands were selected for testing on all 225 individuals in subsequent PCR reactions. The reproducibility of the SSR bands for each pair of primer analyzed was evaluated by repeating the amplification process under the same conditions and observing whether the same pair of primer produced the same amplification result. The reliable pairs of primers identified using this approach is presented in Table 2.

### 2.3. PCR amplification and electrophoresis

PCR amplification was performed in a reaction volume of 10 μL containing approximately 25 ng of the template DNA, 1.0 μL of 10X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 300 nM primers, 0.5 unit of Taq polymerase, and double-distilled water. A reaction mixture without the template DNA was also included in the experiment as the negative control to verify the absence of contamination. The amplification reaction was performed inside a thermal cycler programmed as follows: an initial step at 93 °C for 3 min, then 35 cycles of 30 s at 93 °C, 30 s at 55–58 °C, and 30 s at 72 °C, and a final extension at 72 °C for 5 min. The PCR products were visualized as DNA bands on 18% polyacrylamide gels. The bands that were without contamination and reproducible in different PCR reactions were analyzed further.

### 2.4. Statistical analysis

The PCR results produced various bands, among which only the ones exhibiting consistent and distinct amplification

**Table 1.** Details of the samples from different populations.

| Code | Populations | Number of samples | Latitude (N) | Longitude (E) | Altitude (M) |
|---|---|---|---|---|---|
| FJ | Longyan, FuJian | 25 | 25°3′ | 116°59′ | 433 |
| YN | Funing, YunNan | 25 | 23°29′ | 105°44′ | 1224 |
| GZ | Tongren, GuiZhou | 25 | 26°7′ | 108°46′ | 557 |
| CQ | Xiushan, ChongQing | 25 | 28°16 | 108°54′ | 618 |
| GX | Jinxiu, GuangXi | 25 | 24°6 | 110°12′ | 840 |
| HB | Laifeng, HuBei | 25 | 29°9 | 109°15′ | 684 |
| HN | Huaihua, HuNan | 25 | 26°8 | 109°46′ | 413 |
| JX | Pingxiang, JiangXi | 25 | 27°35 | 114°8′ | 346 |
| GD | Meizhou, GuangDong | 25 | 24°8 | 116°8′ | 202 |

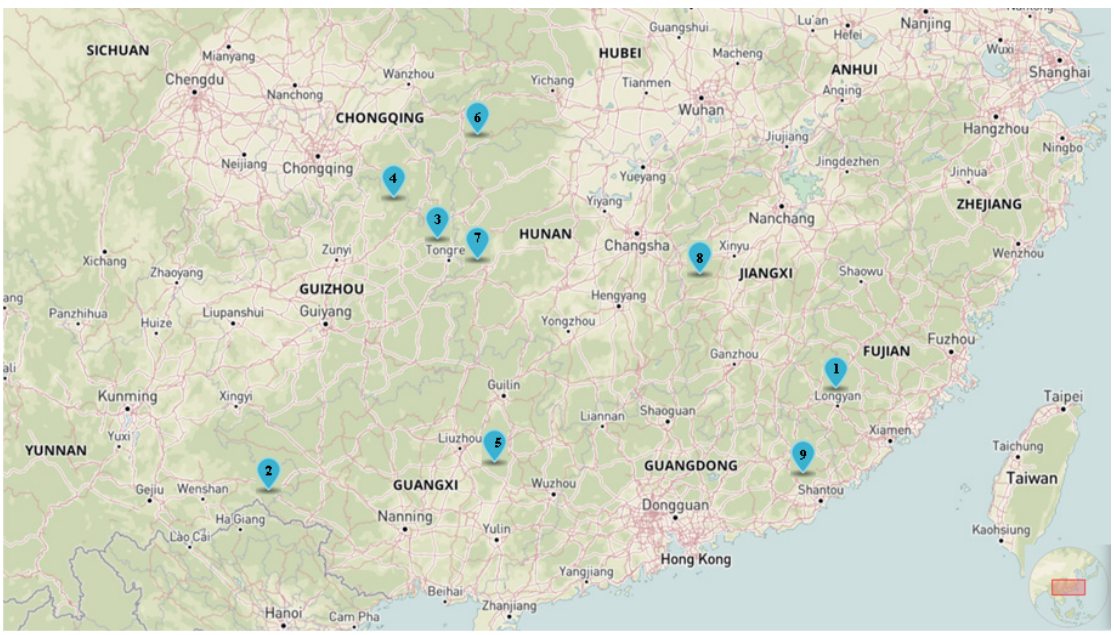


**Figure 1.** Locations from where the *A. grossedentata* populations were sampled. Note: Sites 1, 2, 3, 4, 5, 6, 7, 8, and 9 represent the Codes FJ, YN, GZ, CQ, GX, HB, HN, JX, and GD, respectively.

were considered, while the weak and ambiguous bands were excluded from further analysis. The presence of a PCR band was scored 1, while the absence of a PCR band was scored 0. The band scoring was performed by three different individuals to minimize the error caused by the manual reading of the bands. The pruning of the loci that fulfilled Lynch and Milligan's criterion (Lynch and Milligan,1994) was refrained from throughout the reading process as it could lead to significant bias in the estimation of the parameters of population genetics (Lee et al., 2002). The statistical analysis of the SSR patterns was based on the following assumptions: (1) The SSR DNA fragments behaved as the characteristics of codominant inheritance, the alleles were all present (amplified). The individuals have 40 chromosomes and diploid karyotype (Figure 2); (2) The comigrating DNA fragments represented the homologous loci; (3) The polymorphic loci were inherited in a nuclear (Mendelian) fashion; (4) The populations were in Hardy-Weinberg equilibrium. The observed number of alleles (*Na*), the effective number of alleles (*Ne*), Shannon's information index (*I*), Wright's (1978) fixation index (*Fis*), Nei's (1973) gene diversity (*H*), and observed heterozygosity (*Ho*) (Nei, 1987), and the expected heterozygosity (*He*) were estimated based on microsatellite genotyping and allele frequencies using the computer software package PopGene version 1.31 (Yeh etal., 1999). Allele frequencies

**Table 2.** The information of the pairs of primers amplified using SSR-PCR.

| Primer | SSRs | Forward primer (5'-3') | Reverse primer (5'-3') | No. of alleles | Annealing temperature(°C) | Size range of alleles | *PIC* |
|---|---|---|---|---|---|---|---|
| AG_1 | (AT)8 | ACCAGAAAATCACTAGGCATGAA | ATTTCTTATCCCATTTCATTTGT | 5 | 55 | 156–236 | 0.6631 |
| AG_2 | (AAACC)4 | CGTCTGAATATTAGTGCCGAGAT | TTTTGTTTTGTTTTGGAATTCGT | 6 | 54 | 160–280 | 0.6163 |
| AG_3 | (CT)11 | CCCCAACAAGACTCTCAATGTTA | GAGAGGGAGGGAGATAGAGAGAA | 5 | 54 | 159–269 | 0.6829 |
| AG_4 | (CAC)6 | TGATGACGATGAGGAGAAGAAAT | ATGCCCCAACCCTTTATTTAGTA | 3 | 54 | 136–190 | 0.5767 |
| AG_5 | (AAC)5 | CCTCCTTTCAATTAAACAACCAC | TCTACCTCCATCCCTCATCTACA | 4 | 55 | 148–208 | 0.5836 |
| AG_6 | (AGA)6 | TGCATCCCTTTGAAGAGAATAAA | AGAGCTGGGTTGTGGTAGTCTTT | 7 | 55 | 158–284 | 0.6044 |
| AG_7 | (TTTTG)4 | AGTCTCATGACCGAGTTGTTGTT | TCACCACTTCCAAAAATTAGAGG | 5 | 55 | 149–249 | 0.6760 |
| AG_8 | (TAT)6 | TTGTATATTGGGCTTGTTTGGTT | TTATCAAAGCCCCAAATGAAATA | 4 | 54 | 123–195 | 0.6634 |
| AG_9 | (TCATTA)4 | GATGTCACATGATTTGTAACCGA | GGAGTTGGAGAGATGTTTCCTTT | 7 | 54 | 156–324 | 0.5846 |
| AG_10 | (TCC)6 | TCAAGCTTATACACTCCAGTCCC | TGTGCTTCACTAGACGACACAGT | 6 | 55 | 137–245 | 0.6700 |
| AG_11 | (GA)8 | GTGGACTGTTCTGAGGCTCC | AACCCTCTCTTTTTCCTTCCTTT | 4 | 54 | 157–221 | 0.6663 |
| AG_12 | (TGC)6 | TACTATTCCTCTTGGTGCAAGGA | TTCTACCTTCTCAACTCATTGCTG | 3 | 55 | 154–208 | 0.6115 |
| AG_13 | (CTA)7 | GCTTTCAACATCCACCGTTATAG | GGGGTTTCAACTTGTGAGGTTA | 5 | 60 | 144–249 | 0.6738 |
| AG_14 | (AGGAC)4 | CCATTTAGAGAAGCCACAACCTA | GTCTGAGGGTGAAAGTGAAAAAT | 6 | 53 | 153–273 | 0.6515 |
| AG_15 | (GTG)6 | AGCAGTGGAGGTAGCAACAATAA | TTTTTGTTGGTGGTGGATAGTTC | 6 | 5 | 145–253 | 0.5902 |
| AG_16 | (AGA)5 | AGTTTGGAATTTGAGGAGGAGAG | AATCCCATAGTCCTCCAAATCAT | 5 | 54 | 155–230 | 0.6990 |
| AG_17 | (TCA)8 | TCTCTCCTCTCGCCCTATAAAAC | ACGGATAGGTTTTGAAGAGTGTC | 4 | 53 | 163–256 | 0.6342 |
| AG_18 | (ATTTC)4 | GATCCAGGTACAATGCAAAATGA | TTCGCTATCTCGCTGTCTCTATC | 4 | 55 | 105–185 | 0.6412 |
| AG_19 | (GAGAGG)4 | GGTAGAGGAACAGTTATGGGTCC | ATCTACTCCCCAACCTCACTCTC | 6 | 54 | 95–239 | 0.6090 |
| AG_20 | (AAAAT)4 | GTTGAGACAAATCCAAAGGAATG | AACCGTCTAGCTTTCCTCAAATC | 7 | 55 | 136–276 | 0.6011 |
| AG_21 | (TAT)6 | ACAACTACAACACAAGCCAGGAT | TGCCAAAACAGGAATCTCTACAT | 5 | 55 | 143–233 | 0.6971 |
| AG_22 | (TCA)8 | TCTTCTCTCCTCTCGCCCTATAA | AGGGACTAGACAGCAAGTCCTTC | 7 | 55 | 159–327 | 0.6250 |
| AG_23 | (TTTGAT)4 | GCAAGGTATGGAAGTAGTGATGG | CCGTCGTATACCAGCCAATTAT | 5 | 55 | 140–260 | 0.6227 |
| AG_24 | (CAGTCT)4 | AATTTCTTCTCTTCCTCCACCTG | ATGGGTTGTTGTTAAGGTTGTTG | 4 | 55 | 148–244 | 0.6500 |
| AG_25 | (GCG)7 | CTGAAGCTCTGGACTGTGATGA | GTCAATCCACAACACCGACTC | 3 | 55 | 133–194 | 0.6723 |
| AG_26 | (TTTTCT)4 | AAGCAAAGGGGTTGGTATAAGAG | GCCCTTTGGAGTTGAAATATGAT | 7 | 55 | 141–309 | 0.6310 |
| AG_27 | (GAATCC)4 | ACCACCTCTGTCGATTTCAAGAT | GGGCGTATAATACTCTTGGTGGT | 6 | 55 | 126–270 | 0.6258 |

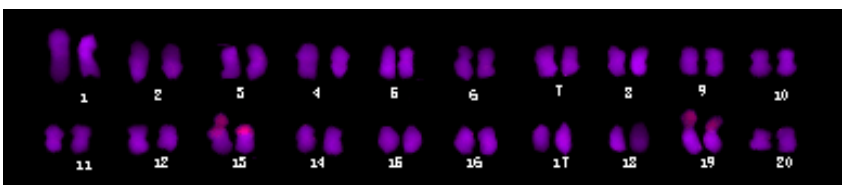


**Figure 2.** The number of chromosomes and karyotype of *A. grossedentata*.

obtained from the microsatellite genotypes were used to calculate *PIC* (polymorphism information content) values (Botstein *et al*., 1980) using the computer software package Cervus 3.0 (Marshall et al., 1998; Kalinowski et al., 2007) in order to measure the information obtained by a microsatellite. In addition, Nei's (1972) original measures of genetic identity and genetic distance were estimated. The genetic structure of the populations and the proportion of genetic divergence within and among the populations were estimated as described by Nei (Nei, 1973). $G_{ST}$ was designated as the coefficient of genetic differentiation among the populations. The genetic

identity ($G_I$) and the genetic distance ($G_D$) among the populations were computed using the model reported by Nei (Barrett and Kohn, 1991). Gene flow estimates (*Nm*) were calculated using the formula $Nm = (1 - G_{ST})/4G_{ST}$ (Slatkin and Barton, 1989). Differentiation among the populations was analyzed by performing the analysis of molecular variance (AMOVA) using GenALEx Version 6.1 with 999 permutations (Peakall and Smouse, 2006). An unweighted pair group method based on the arithmetic average (UPGMA) dendrogram was applied on the data matrix of the mean character differences between the pairs of samples using the software MEGA version 6 (Tamura et al., 2013). The relationship between the latitude and longitude and the molecular indices was calculated as a two-polynomial regression. Correlations were calculated using the software SAS version 6.12 (SAS Institute Inc., 1996). The program STRUCTURE (Pritchard et al., 2007) was employed to ascertain a possible cryptic genetic structure in the dataset analyzed in this experiment. When operating the program, the natural logarithm of the probability that a given genotype X is a part of a given population K was used for inferring the population structure using the Markov chain Monte Carlo method (McMC). McMC is based on the assumption of Hardy-Weinberg equilibrium and attempts to identify the population groupings that are not in linkage or Hardy-Weinberg disequilibrium. In order to quantify the variation in the probability for each number of clusters (K), a series of 10 independent runs were performed for each value of K ranging from 1 to 20, assuming an admixture model with independent allele frequencies and using a burn-in period of 1,000,000 iterations and a data collection period of 1,000,000 iterations. The sdL(K) and mean |L"(K)| values computed for each K were processed using an ad hoc method (Evanno et al., 2005) to calculate the Mean LnP(K), Stdev LnP(K), and Delta K values, which would indicate the true K value. The genetic relationships among all the individuals sampled from different populations were subjected to the principal coordinates analysis (PCoA) to visually represent the relationships between the populations and to assess the distinctiveness of all populations, which would allow determining whether there were distinct genetic groupings among the populations. The program GenAlEx version 6.1 was employed for PCoA.

## 3. Results

### 3.1. Genetic diversity

The selected 27 pairs of primers were used for amplifying 225 individuals from 9 populations of *A. grossedentata*. Table 3 details the molecular variation in this species as revealed by the SSR analysis. The observed number of alleles (*Na*) was 5.1111 at the species level, while the number ranged from 4.3704 to 4.7037 at the population level. The effective number of alleles (*Ne*) was 3.9965 at the species level, while the number ranged from 2.1713 to 3.3444 at the population level. Shannon's information index (*I*) was determined to be 1.4506 at the species level, and the index value ranged from 1.1942 to 1.2969 at the population level. The observed heterozygosity (*Ho*) was 0.7111 at the species level, and the value at the population level ranged from 0.6548 to 0.8430. The expected heterozygosity (*He*) was 0.7356 at the species level, and the value at the population level ranged from 0.6566 to 0.6944.

### 3.2. Genetic differentiation

The genetic differentiation among the nine populations was estimated at the level of 27 microsatellite loci. All loci were polymorphic in the nine populations analyzed. The F-statistics for all 27 loci were used for analyzing the

**Table 3.** Molecular variation measured as the observed number of alleles (*Na*), the effective number of alleles (*Ne*), Shannon's information index (*I*), observed heterozygosity (*Ho*), and expected heterozygosity (*He*) along with their standard deviation (S.D.) values. The population-specific genotype (*PSG*) values are also provided.

| Pop. ID | *Na* (S.D.) | *Ne* (S.D.) | *I* (S.D.) | *Ho* (S.D.) | *He* (S.D.) | *PSG* |
|---|---|---|---|---|---|---|
| CQ | 4.3704 (1.0432) | 2.1713 (0.7587) | 1.1942 (0.2515) | 0.8430 (0.2054) | 0.6566 (0.1097) | 0 |
| GZ | 4.5185 (0.9755) | 3.2656 (0.7345) | 1.2785 (0.2151) | 0.8302 (0.1515) | 0.6920 (0.0786) | 0 |
| HN | 4.6667 (1.1435) | 3.3444 (0.9060) | 1.2969 (0.2513) | 0.7689 (0.1702) | 0.6944 (0.0847) | 5 |
| JX | 4.3704 (1.1145) | 3.0225 (0.8144) | 1.2006 (0.2672) | 0.7719 (0.2125) | 0.6577 (0.1031) | 2 |
| HB | 4.5185 (1.1559) | 3.1609 (0.7650) | 1.2548 (0.2337) | 0.7244 (0.2217) | 0.6775 (0.0923) | 4 |
| YN | 4.5926 (1.2172) | 3.1533 (0.7833) | 1.2600 (0.2283) | 0.7141 (0.1895) | 0.6794 (0.0748) | 2 |
| FJ | 4.5556 (0.9740) | 3.2346 (0.7586) | 1.2766 (0.2224) | 0.6548 (0.1332) | 0.6875 (0.0811) | 4 |
| GX | 4.7037 (1.1373) | 3.0790 (0.6101) | 1.2574 (0.2010) | 0.6652 (0.2282) | 0.6759 (0.0708) | 8 |
| GD | 4.5926 (1.1184) | 3.2616 (0.6427) | 1.2812 (0.1978) | 0.7111 (0.1939) | 0.6938 (0.0750) | 5 |
| Total | 5.1111 (1.2810) | 3.9965 (1.0117) | 1.4506 (0.2408) | 0.7422 (0.1294) | 0.7356 (0.0674) | 30 |

population genetic structure. Highly significant genetic divergence across the nine populations was observed for every locus. The F-statistics and the gene flow for all loci are presented in Table 4. The average value of the Wright's fixation index (*Fis*) was –0.1154, with the range of –0.4627 to 0.2399, at the population level. The *Fis* value of five loci, namely, AG_3, AG_15, AG_16, AG_19, and AG_27, was less than zero, which indicated that their heterozygotes were not sufficient. The others (loci) appeared with heterozygous excess. The average *Fit* value was –0.0119, with the range of –0.3523 to 0.3563, at the population level. The *Fst* value was 0.0928 on average, ranging from 0.0463 (AG_11) to 0.1567 (AG_16). Using the multilocus *Fst* values, approximately 9.0% of the total genetic variation could be explained by breed differences, while the remaining 91.0% was due to the natural differences among the individuals (Table 4). There were no highly differentiated loci (*Fst*>0.25). Only two loci AG_16 and AG_21 exhibited a large genetic differentiation among the populations (0.15 < *Fst* < 0.25). Most of the loci exhibited a moderate degree of genetic differentiation among the populations (0.05 < *Fst* < 0.15). The AG_11 locus exhibited extremely small genetic differentiation (*Fst* < 0.05), which could be ignored. In comparison to the genetic differentiation among populations, the genetic variation within the populations was significantly larger, with an average gene flow value of 2.4426 (*Nm*>1). The deviation from the Hardy-Weinberg equilibrium (HWE) between the loci and the populations was evaluated using FSTAT (version 2.9.3, Goudet, 2001). The significance levels

**Table 4.** Summary of the F-statistics and gene flow for all loci.

| Locus | *Fis* | *Fit* | *Fst* | *Nm* |
|---|---|---|---|---|
| AG_1 | –0.0348 | 0.0617 | 0.0932 | 2.4325 |
| AG_2 | –0.2087 | –0.0966 | 0.0928 | 2.4454 |
| AG_3 | 0.0626 | 0.1601 | 0.1040 | 2.1533 |
| AG_4 | –0.0170 | 0.0412 | 0.0572 | 4.1188 |
| AG_5 | –0.4225 | –0.2927 | 0.0913 | 2.4890 |
| AG_6 | –0.2236 | –0.1301 | 0.0765 | 3.0199 |
| AG_7 | –0.1334 | –0.0424 | 0.0803 | 2.8637 |
| AG_8 | –0.1465 | –0.0344 | 0.0978 | 2.3069 |
| AG_9 | –0.1475 | –0.0209 | 0.1103 | 2.0159 |
| AG_10 | –0.0006 | 0.0837 | 0.0842 | 2.7177 |
| AG_11 | –0.2356 | –0.1784 | 0.0463 | 5.1475 |
| AG_12 | –0.2306 | –0.1408 | 0.0730 | 3.1764 |
| AG_13 | –0.3562 | –0.2280 | 0.0945 | 2.3942 |
| AG_14 | –0.2354 | –0.1423 | 0.0753 | –3.0704 |
| AG_15 | 0.0658 | 0.1748 | 0.1167 | 1.8926 |
| AG_16 | 0.1465 | 0.2803 | 0.1567 | 1.3451 |
| AG_17 | –0.1042 | 0.0277 | 0.1194 | 1.8434 |
| AG_18 | –0.1753 | –0.0496 | 0.1070 | 2.0870 |
| AG_19 | 0.1250 | 0.1988 | 0.0844 | 2.7133 |
| AG_20 | –0.1416 | –0.0456 | 0.0841 | 2.7238 |
| AG_21 | 0.2399 | 0.3563 | 0.1530 | 1.3836 |
| AG_22 | –0.1114 | 0.0021 | 0.1021 | 2.1982 |
| AG_23 | –0.0876 | 0.0116 | 0.0912 | 2.4898 |
| AG_24 | –0.2052 | –0.1321 | 0.0607 | 3.8681 |
| AG_25 | –0.4627 | –0.3523 | 0.0755 | 3.0612 |
| AG_26 | –0.1310 | –0.0406 | 0.0799 | 2.8807 |
| AG_27 | 0.0694 | 0.1523 | 0.0891 | 2.5554 |
| Mean | –0.1154 | –0.0119 | 0.0928 | 2.4426 |

were adjusted using Bonferroni correction for multiple evaluations.

Table 5 presents an estimate of Nei's genetic identities ($G_I$) and genetic distance ($G_D$) for each pairwise comparison between two populations. The $G_D$ values between the populations varied from 0.0426 to 0.4311, while the $G_I$ values between the populations varied from 0.6498 to 0.9583 (Table 5 for details). The correlation analysis conducted using the statistical analysis system (SAS 9.4) revealed no significant correlation either between genetic differentiation and latitude ($p>0.05$) or between genetic differentiation and longitude ($p>0.05$).

The AMOVA results indicated that most (66.34%) of the molecular variation in the *A. grossedentata* populations analyzed existed within the populations, while lesser amounts of variation (33.66%) existed among the populations (Table 6).

A UPGMA dendrogram constructed based on Nei's (1972) genetic distance indicated that the nine populations analyzed were clustered into the following five geographically distinct groups (Figure 3): South China (GD), Southeast China (FJ), Central South China (HN, JX, and HB), Southwest China (YN and GX), and West China (CQ and GZ). The results of the Mantel tests revealed no significant association between the genetic distance and the geographic distance among the studied populations of *A. grossedentata*.

The STRUCTURE analysis detected two genetic clusters (Figure 4). At K = 2, the individual assignments in the two clusters remained consistent in 10 replicate runs. The GD, FJ, and GX populations belonged to one cluster, while the remaining populations (CQ, GZ, YN, HN, HB, and JX) belonged to the other cluster.

The result of the principal coordinates analysis showed the GD population belonged to one cluster, The GX population belonged to one cluster, while the remaining populations (CQ, GZ, YN, HN, HB, JX and FJ) belonged to the other cluster (Figure 5).

## 4. Discussion

### 4.1. Genetic diversity in *A. grossedentata*

The evolution rate of the SSR sequences is higher than that of most other types of DNA sequences, thereby allowing for a higher degree of variation. Therefore, SSR markers are able to detect the differences in several loci in the genome, thereby exhibiting high polymorphism (Bell and Ecker, 1994; Botta R et al., 1999). Meanwhile, the SSR analysis involves the use of a long primer that is 16–25 bp in length, because of which it requires a higher annealing temperature and exhibits high precision and good repeatability in PCR amplification. The SSR marker technology is simple and reliable with high polymorphism and good repeatability, which renders this technique effective for evaluating the genetic diversity of *A. grossedentata*.

Nybom and Bartish (2000) believed that expected heterozygosity (*He*) and Shannon's information index (*I*) were more important when analyzing the genetic diversity, and they, therefore, focused on the genetic diversity of the species at the population level. The results of the present study revealed that *A. grossedentata* was rich in genetic diversity and maintained a high genetic variation which could be attributed to its biological characteristics. Firstly, according to the investigation conducted by our research group, *A. grossedentata* is distributed widely across China, which results in abundant genetic variation in this species due to natural selection. Secondly, *A. grossedentata* is mainly a wild species currently with negligible human intervention in the selection process, thereby resulting in a lower loss in the diversity characters.

According to the results of the present study, the HN, GD, GZ, FJ, JX, HB, YN, and GX populations presented a higher value of Nei' genetic diversity index (*He*) and Shannon's information index (*I*), which indicated that these populations were rich in genetic diversity and provided a good basis for breeding. The germplasms of these populations are, therefore, important resources

**Table 5.** Nei's (1972) original measures of genetic identity (above the diagonal) and genetic distance (below the diagonal).

| Pop. ID | CQ | GZ | HN | JX | HB | YN | FJ | GX | GD |
|---|---|---|---|---|---|---|---|---|---|
| CQ | - | 0.9583 | 0.9068 | 0.8274 | 0.8034 | 0.8689 | 0.8770 | 0.6586 | 0.7009 |
| GZ | 0.0426 | - | 0.8924 | 0.8314 | 0.8259 | 0.8431 | 0.8772 | 0.6801 | 0.7258 |
| HN | 0.0978 | 0.1138 | - | 0.8114 | 0.7708 | 0.8158 | 0.8048 | 0.6498 | 0.7262 |
| JX | 0.1895 | 0.1846 | 0.2090 | - | 0.7535 | 0.8104 | 0.7992 | 0.7306 | 0.7485 |
| HB | 0.2189 | 0.1913 | 0.2604 | 0.2831 | - | 0.7342 | 0.7524 | 0.6594 | 0.6878 |
| YN | 0.1405 | 0.1707 | 0.2036 | 0.2102 | 0.3090 | - | 0.7846 | 0.6687 | 0.6855 |
| FJ | 0.1312 | 0.1310 | 0.2171 | 0.2241 | 0.2846 | 0.2426 | - | 0.6743 | 0.7122 |
| GX | 0.4177 | 0.3855 | 0.4311 | 0.3139 | 0.4165 | 0.4025 | 0.3941 | - | 0.6880 |
| GD | 0.3554 | 0.3205 | 0.3199 | 0.2897 | 0.3742 | 0.3776 | 0.3394 | 0.3739 | - |

**Table 6.** Analysis of molecular variances for the nine *A. grossedentata* populations analyzed.

| Source of variation | Degrees of freedom | Sum of squares | Variance components | Total variance (%) |
|---|---|---|---|---|
| Among populations | 8 | 1447.077 | 3.49549 | 33.66 |
| Among individuals Within populations | 215 | 765.352 | 1.80327 | 20.18 |
| Within individuals | 224 | 2259.500 | 5.08705 | 46.16 |
| Total | 447 | 4471.929 | 10.38581 | 100 |
| Fixation index | Value | | | |
| FST | 0.3366 | | | |

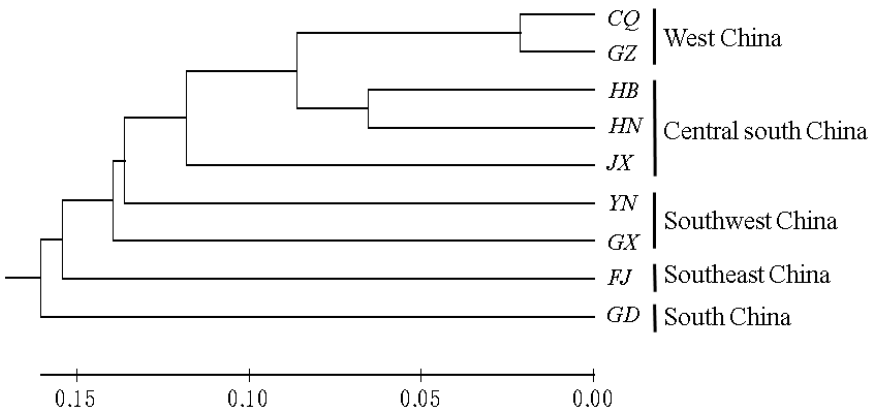


**Figure 3.** UPGMA dendrogram based on Nei's (1972) genetic distance.

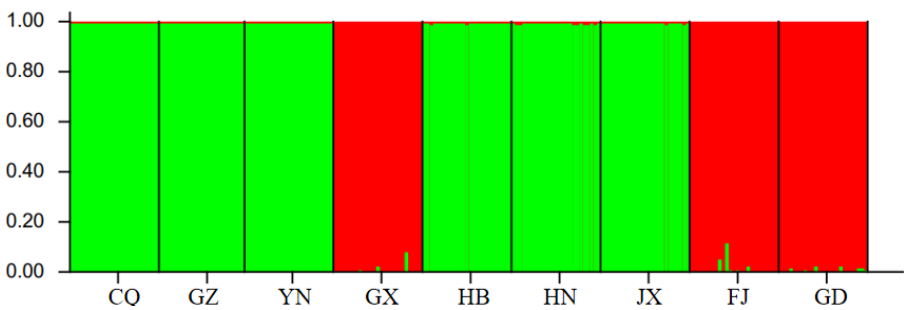


**Figure 4.** Individual assignment based on the STRUCTURE analysis. Bold black lines within the squares distinguish the populations. Abbreviations for each population name are provided under the bars.

for the research and genetic breeding of *Ampelopsis grossedentata*. The genetic diversity of the CQ population was relatively low.

That the specific bands displayed in the different population could form a specific genotype in individuals, which is affected by various factors in the evolution process. The complex genetic composition was reflected in its genetic structure. These factors included mutation, population history, genetic drift, natural selection, recombination, etc. They had different effects on the genetic composition of the population. The specific genotypes showed the diversity and complexity of genetic background. It might be an adaptive performance of species against adverse environmental factors in the process of evolution, and had important biological significance to maintain the survival and continuation of the population. On the other hand, when the same species those had specific genotypes were separated due to geographical constraints, such as mountains or oceans, they might begin to evolve independently. It could evolve

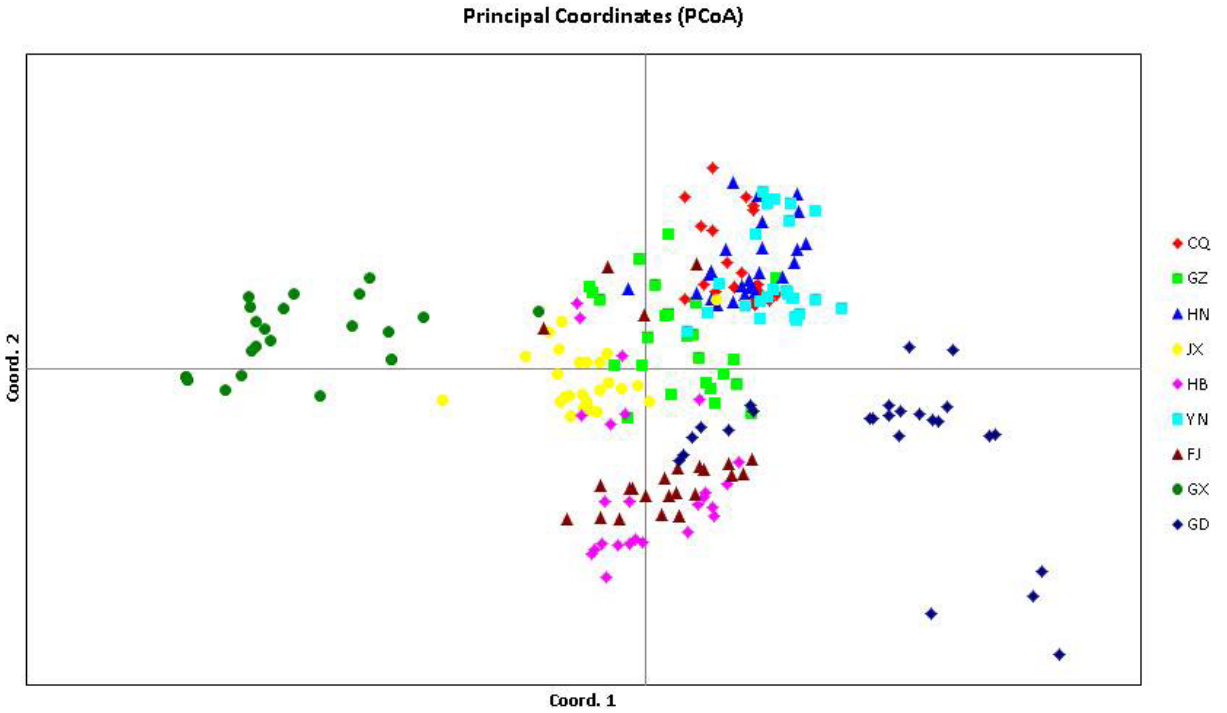


**Figure 5.** Results of the principal coordinates analysis.

into a similar "sister species" in the future, and as time went on, the genetic differences between the two independently evolved species would become more and more obvious. That is, when the same gene rapidly evolved in two similar species, they would become different and could no longer be compatible with each other. As Darwin predicted before, they would continue to evolve and differentiate under natural selection.

### 4.2. Population genetic structure and differentiation of *A. grossedentata*

Population genetic structure, which is one of the most basic characteristics of a species, is reflected by the genetic differentiation between and within populations. Since the genetic structure is affected by the selection process, the mating system, gene flow, and genetic drift, the population genetic structure is often studied based on the population genetic differentiation and gene flow. Several factors affect the genetic differentiation of natural populations, among which gene flow, gene mutation, breeding system, seed diffusion and distribution, and the selection of the natural environment are the most important ones (Loveless and Hamrick,1984; Wei and Wu, 2012; Duan et al., 2021).

In a sense, the population genetic structure is a consequence of the interaction between gene flow and genetic drift (Hutchison et al., 1999). It is generally believed that the population genetic differentiation caused by the genetic drift is preventable if the gene flow value (Nm) equals 1 ( Slatkin, 1987). The results of the present study demonstrated that the average Nm of *A. grossedentata* population was 2.4426, i.e. Nm was greater than 1, which indicated that the gene flow could resist the population differentiation caused by genetic drift. The gene flow of *A. grossedentata* observed in the present study was significantly higher than that of the cross-pollinated plants, which indicated that the gene flow of *A. grossedentata* was relatively high. The reasons for this could be as follows: (1) The breeding system of *A. grossedentata* is characterized by outcrossing, which leads to a relatively high gene flow value for *A. grossedentata*; (2) The population of *A. grossedentata,* according to field surveys, was mostly distributed in the form of dots and fragments, with the distance of over 500 m between each sample in the experimental population, which led to the relatively high gene flow; (3) The main pollinators of *A. grossedentata* are insects and wind vectors, and *A. grossedentata* blooms in summer, a period of several insects and strong wind, which leads to high natural sexual reproduction rate, resulting in a relatively high gene flow; (4) There is no evidence that geographic isolation hinders the gene exchange among different populations.

Furthermore, although the genetic variation of *A. grossedentata* was similar within and among the populations, the genetic variation within the populations was higher than the genetic variation among different populations. Theoretically, compared to asexual plants, sexual plants present a relatively higher level of genetic variation while the level of genetic variation among the

populations is decreased, particularly in purely sexual plants. The field investigation and research conducted by our research group revealed that the fruit setting rate in *Ampelopsis grossedentata* was higher in the natural reproduction system, which improved the genetic variation level within the population while reducing the differences among the populations. These reasons explain the higher genetic variation within the populations compared to that among the populations.

**4.3. Implication for taxonomy**

The results of the UPGMA analysis of different populations revealed that the populations of *A. grossedentata* were categorized into five branches, among which the respective genetic distances of GD and FJ from the other populations were the largest, and the remaining branches were formed by YN and JX, HN and HB, and GZ and CQ. According to the UPGMA tree, GD was the origin of *A. grossedentata*, and it was divided into two branches: the northeast branch and the northwest branch. The northeast branch extended from the south China GD to the southeast China FJ to the JX, HN, HB, and other populations from the central south China regions. The northwest branch extended from the southern China GD to the west China GZ and CQ to the GX and YN from the southwest China region. According to the geographical distribution and the relative associations demonstrated in the UPGMA tree, the species of *A. grossedentata* could survive in the last Pleistocene glacial period in the Meizhou and Guangdong regions of South China, extending further to the northwest and northeast of the southwest region, based on which the present distribution pattern appeared. The genetic variation in the population could mainly be caused by the subsequent gene flow and natural selection. The gene flow altered the distribution of genetic variation by either increasing the variation or reducing the differentiation among the populations. Although according to the UPGMA map, the distribution of *A. grossedentata* was generally from the south to the northeast and northwest, no significant correlation was observed between genetic distance and geographical distance to support the hypothesis of this historical relationship, and only the subsequent role of gene flow could explain the previously-stated variation pattern. According to the structure analysis, the nine populations of the individuals sampled were categorized into two clusters (Figure 3). The three populations from south China (GD), southeast China (FJ), and southwest China (GX) were classified together as one cluster, while the remaining populations were classified as the second cluster. The obtained genetic clusters were roughly consistent with the results of the UPGMA analysis. The result of the principal coordinates analysis showed the GD population belonged to one cluster, the GX population belonged to one cluster, while the remaining populations (CQ, GZ, YN, HN, HB, JX and FJ) belonged to the other cluster (Figure 5). It was further proved that this species originated from South China and was divided into two branches extending southeast and southwest of China, which showed the PCoA clustering results were the same as the UPGMA clustering results and were basically consistent with the results obtained by the Structure software based on the data.

**4.4. Protection, development, and utilization of the wild resources of *A. grossedentata***

The dry roots, stems, and leaves of *A. grossedentata* are referred to as 'Tian Cha Teng' when used as medicinal materials. With the increasing usage of 'Tian Cha Teng' every year, the natural regeneration speed of *A. grossedentata* has decelerated. While the wild resources of *A. grossedentata* have already been seriously damaged due to greater excavation, even the current levels of artificial cultivation of the species have failed to meet the large-scale cultivation requirements. Meanwhile, the destruction of the ecological environment and the extensive use of pesticides are increasingly reducing the habitat suitable for the effective growth of *A. grossedentata*. The above phenomena would eventually cause a continuous reduction and degradation of the genetic diversity of the germplasm resources of this species. The research on plant genetic diversity forms the basis for the protection, rational utilization, and genetic improvement of a species. The level of the genetic diversity of a species reflects, to a certain extent, the ability of that species to adapt to its environment and, therefore, limits its level of adaptive evolution. Moreover, the level of genetic diversity of a species provides crucial information for evaluating the current situation and the protection value of the species. In the present study, SSR molecular markers were utilized to analyze the genetic diversity and the genetic relationship of *A. grossedentata*. The findings revealed the genetic relationship of the germplasm resources of *A. grossedentata* at the molecular level, thereby providing a scientific basis for breeding, quality evaluation, resource protection, and utilization in the future. The present study revealed that *A. grossedentata* was rich in genetic diversity. Therefore, it is imperative to formulate practical strategies to protect the genetic resources of *A. grossedentata* and accelerate the industrialization process of the artificial cultivation technology of *A. grossedentata*. Wild tending, artificial pollination, and protection of the ecological environment would allow the gradual expansion of the population number and scale of *A. grossedentata.* Research on the key technologies of artificial cultivation and breeding of excellent varieties would popularize the technology of the artificial cultivation of *A. grossedentata*. Therefore, through artificial cultivation technology, it would be possible to meet the increasing market demand for *A. grossedentata*,

which would eliminate the current requirement of relying on its wild resources. *A. grossedentata* is an important medicinal herb for the ethnic minorities of China; therefore, multiple protection measures should be adopted for its effective conservation. The natural populations of *A. grossedentata* should be subjected to in situ conservation measures, such as setting aside natural conservation areas in Guangdong (GD), Fujian (FJ), and Guangxi (GX), as well as to ex situ conservation measures involving the introduction of germplasm resources from additional natural places, if feasible, to maintain as much genetic diversity as possible. In addition, it must be ensured that the approaches used for the protection, development, and utilization of the genetic resources of *A. grossedentata* are sustainable in nature.

## 5. Conclusion

In conclusion, the genetic structure of the populations and the levels of genetic diversity of the species observed in the present study could provide crucial information for evaluating the current situation and the protection value of this species. The results revealed that *A. grossedentata* germplasm resources was rich in genetic diversity and the present distribution pattern of *A. grossedentata* was generally from the south region to the northeast and northwest regions in China. The southern region could become a conservation center for this species. Therefore, on the one hand, the natural populations of *A. grossedentata* should be subjected to in situ conservation measures, setting aside natural conservation areas in the southern region in China, as well as to ex situ conservation measures, involving the introduction of germplasm resources from additional natural places, if feasible, to maintain as much genetic diversity as possible. On the other hand, it should be imperative to formulate practical strategies to protect the genetic resources of *A. grossedentata* and accelerate the industrialization process of the artificial cultivation technology of *A. grossedentata*. It would be possible to meet the increasing market demand for *A. grossedentata*.

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