

DNA Barcode Reference Library Construction and Genetic Diversity and Structure Analysis of *Amomum Villosum* Lour. Populations in Guangdong Province

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Research

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Abstract

Background

Amomum villosum Lour. is the plant of a famous traditional Chinese medicine Amomi Fructus and its habitat has been frequently destroyed that seriously threatened its germplasm resources. The genetic diversity has great significance to the optimization of germplasm resources and protection of populations, but that of *A. villosum* is unclear. In this study, we analyzed the genetic diversity and genetic structures of *A. villosum* populations in Guangdong and constructed a local reference DNA barcode library for the purpose of more appropriate protecting measures.

Methods

DNA barcoding and ISSR markers were used to investigate the population genetics of *A. villosum*. Five universal DNA barcodes were amplified and constructed a DNA barcode reference library. Parameters including percentage of polymorphic sites (PPB), number of alleles (N_a), effective number of alleles (N_e), Nei's gene diversity index (H) and Shannon's polymorphism information index (I) were calculated for the assessment of genetic diversity. Genetic structure was revealed by Nei's gene differentiation coefficient (G_{st}), total population genetic diversity (H_t), intra-group genetic diversity (H_s) and gene flow (N_m). AMOVA analysis, Mantel tests, UPGMA dendrogram and PCoA analysis were used to elucidate the genetic differentiation and the relationship among populations.

Results

531 sequences of five DNA barcodes were gained and all the sequences of the each barcode had no variation sites. A total of 66 ISSR bands were generated from *A. villosum* populations by the selected 6 ISSR primers. And 56 bands were amplified polymorphic with the percentage was 84.85% for all the seven *A. villosum* populations. The *A. villosum* populations showed high genetic diversity ($H = 0.3281$, $I = 0.4895$) whereas the gene flow was weak ($N_m = 0.6143$). G_{st} (0.4487) and AMOVA analysis indicated that there's obvious genetic differentiation among *A. villosum* populations and more genetic variations existed in within population. The genetic relationship of each population was relatively close as the genetic distances were between 0.0844 and 0.3347.

Conclusions

Overall, this study confirmed a high genetic diversity but weak gene flow of *A. villosum* in Guangdong province, especially in Amomi Fructus's Daodi producing area, Yangchun city. And a local DNA barcode reference library containing 531 sequences were subsequently constructed; facilitating the identification, conservation, breeding and cultivation of *A. villosum*.

Introduction

Amomum villosum Lour. is a medicinal plant of Zingiberaceae family mainly grown in southern China. Its ripe and dried fruit Amomi Fructus is the famous traditional Chinese medicine (TCM) with the effects of dampening appetite, warming the spleen to stop diarrhea, regulating qi and relieving the fetus. Modern pharmacological studies show that, Amomi Fructus has great activities of anti-ulceration, anti-diarrhea, anti-inflammatory and antimicrobial [1]. In addition, Amomi Fructus is also widely used in food, liquors, and tea as the health product and condiment. Yangchun City located in Guangdong Province is considered the Daodi (genuine) producing area of Amomi Fructus for its high quality. With the rapid development of the city as well as traditional Chinese medicine business, the habitat of *A. villosum* has been frequently destroyed that seriously threatened its germplasm resources [2]. In 2016, Amomi Fructus from Yangchun was selected as one of the eight legally protected TCM varieties in Guangdong Province [3].

The genetic diversity of a species is the basis for its survival and evolution, which is of great significance to the analysis of evolutionary polymorphism, genetic relationship, and optimization of germplasm resources and protection of populations. Polymerase Chain Reaction (PCR)-based molecular markers have been widely used in the analysis of plant genetic diversity. Among them, Inter-Simple Sequence Repeat (ISSR) is fast and efficient, with the characteristics of high polymorphism, high reliability, low cost, and does not require pre-determination of target sequence information [4, 5]. Another marker, DNA barcodes, proposed in 2003 can not only be used in biological identification, but also in genetic diversity analysis [6, 7].

COI was an efficient species identification tool for animals and frequently used in genetic diversity analysis of animals [8]. In plants, however, low substitution rates of mitochondrial DNA have made it unsuitable, and some other barcoding regions were researched as alternatives [9]. In the current study, ISSR and five DNA barcodes ITS2, *psbA-trnH*, ITS, *matK* and *rbcl* were used to investigate the genetic diversity of seven populations of *A. villosum* in Guangdong Province, especially in the Daodi producing area of Amomi Fructus, Yangchun City. It will provide insights into the identification, conservation, breeding and cultivation of *A. villosum*.

Materials And Methods

Plant Material Sampling

A total of 141 samples of 7 *A. villosum* populations were collected in Guangdong Province from August to November 2018. Six of them were in Yangchun City and the other one was in Maoming City. Population ZY was collected from a deserted germplasm garden containing *A. villosum* plants coming from Guangdong (Jinhuakeng), Yunnan and Guangxi provinces and a foreign country Myanmar. The sampled plants were identified by Huang Zhihai, the chief Chinese pharmacist of the Second Clinical College of Guangzhou University of Chinese Medicine. Fresh and healthy leaves were removed from the

plants, dried and preserved in silica gel right now in the field, then stored in an ultra-low temperature refrigerator (Eppendorf, Hamburg, Germany) when came back to the laboratory. Detailed information and geographic location of the samples see Table 1 and Fig. 1.


DNA extraction, PCR amplification and sequencing

The total DNA was extracted using a DP305 plant DNA kit (TIANGEN Biotech Co., Ltd., Beijing, China). The NanoDrop2000 ultra-micro ultraviolet spectrophotometer (Thermo Scientific, MIT, USA) was used to determine the DNA concentration and purity. The PCR amplification reaction system of the experiment contained 2×Taq PCR Mix 12.5 µL, forward primer (2.5 µM) 1.0 µL, reverse primer (2.5 µM) 1.0 µL, genomic DNA 2.0 µL and added up to 25 µL with ddH₂O. The primer sequences and amplification conditions of different DNA barcodes were shown in Table S1. All amplification reactions were completed on the ProFlex PCR instrument (Life Technologies, New York, USA). PCR products were sent to Shanghai Majorbio Pharmaceutical Biotechnology Co., Ltd Guangzhou Branch to be sequenced.

ISSR-PCR amplification system

100 ISSR universal primer sequences published by Columbia University were screened [10]. And 6 primers that produced clear and reproducible banding patterns were selected (Table 3). ISSR-PCR amplifications were performed on 38 individuals randomly chosen from seven *A. villosum* populations (Table 4). The 20µL ISSR reaction volume including 10µL2X PCR Mix (containing dye, MgCl₂, dNTPs), 2µL template DNA, 1µLISSR selected primer and 7µL ddH₂O. The ISSR PCR amplification was programmed in the ProFlex thermocycler as follows: predenaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 45s, annealing at the 46.83 - 56.60 °C of the primer for 45s, extension at 72 °C for 2min, with a final extension at 72 °C for 5 min and preservation at 4 °C. ISSR-PCR products were separated on a 2% agarose gel stained with Goldview by electrophoresis in 1×TAE buffer at 80 V. The gels were visualized under UV light and photographed with a ChemiDoc imaging system (Bio-Rad, California, USA). The molecular weights of ISSR-PCR products were estimated using a 100bp Plus DNA Ladder (TIANGEN Biotech Co., Ltd., Beijing, China).

Data analysis

The two-way sequenced peaks of DNA barcodes were evaluated and assembled by CondonCode Aligner v8.0.1 software [11]. Low-quality areas at both ends of the assembled sequences were removed. ITS2 barcodes were annotated by cutting off the conserved 5.8S and 28S motifs based on HMM [12] at the ITS2 database [13]. Mega6.0 software was used to align DNA barcode sequences and calculate sequence statistics including the base composition ratio, GC content, heterotopic site information, conservative site and parsimony informative sites [14]. Haplotype sequences for each barcodes were exhibited in the two-dimensional code picture. In the picture, each vertical line represented a nucleotide base (), and the two-dimensional code on the right could be scanned directly with the DNA sequence.

Reproducible ISSR-PCR bands were determined with the help of the GelPro32 software and manual correction. These clear bands were scored as either present (1) or absent (0), thus generating an ISSR phenotype data matrix. And the data matrix was imported in Popgene32 software to analyze genetic diversity and genetic structure [15]. Genetic diversity parameters included percentage of polymorphic sites (PPB), number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity index (H) and Shannon's polymorphism information index (I) were calculated. Genetic structure parameters including Nei's gene differentiation coefficient (Gst), total population genetic diversity (Ht), intra-group genetic diversity (Hs) and gene flow (Nm) were calculated. GenAlEx 6.502 software was used to estimate the components of genetic variance within and among populations by analysis of molecular variance (AMOVA) and to assess the correlation between population genetic distance and geographic distance by Mantel tests [16]. Genetic distances among populations were calculated and a UPGMA dendrogram was constructed by using NTSYS 2.10e [17].

Results And Analysis

DNA barcode reference library construction

We extracted the genomic DNA from 141 samples of *A. villosum*. The OD_{260/280} was 1.76-1.98 for all the DNA samples and the concentration was 73.70-1294.80 ng/μL. Five DNA barcodes of all the samples were amplified and sequenced bi-directionally. The PCR amplification and sequencing results were shown in Table 2. It showed that the success rate of sequencing for each barcode was *rbcl* (100.00%) > ITS (98.58%) > ITS2 (95.04%) > *psbA-trnH* (53.90%) > *matK* (29.08%). The ranking of the success rate of PCR amplification was consistent with that of sequencing. Thus, these sequences constructed the DNA barcode reference library of *A. villosum* in Guangdong Province.

We also analyzed the sequences of each barcode we obtained. All the sequences of the five barcodes had no variation sites, showing a strong conservation. Among the five DNA barcodes, ITS2 had the shortest sequence length and the highest GC content. The sequences of each barcode were shown in Fig. 2.

ISSR polymorphism and genetic diversity

We established the ISSR-PCR system for *A. villosum*. The ISSR-PCR fragments of *A. villosum* populations ranged from 300bp to 2000 bp. An example of amplified profiles was shown in Fig. S1. A total of 66 ISSR bands were generated from *A. villosum* populations by the selected 6 primers. Among them, 56 bands were polymorphic, thus the percentage of polymorphic bands was 84.85% for all the seven *A. villosum* populations. For each primer, it amplified 6-14 bands with the polymorphic ratio was 64.29%-100.0%. The results were shown in Table 3.

Genetic diversity information of *A. villosum* populations analyzed by Popgene 32 was shown in Table 4. Among the seven populations of *A. villosum*, population ZY had the highest genetic diversity (PPB=71.21%, Na=1.7121, Ne=1.4240, H=0.2536, I=0.3816) while population TK had the lowest genetic

diversity (PPB=28.79%, Na=1.2879, Ne=1.1848, H=0.1117, I=0.1658). At the species level, the value of Na, Ne, H and I were 1.9394, 1.5662, 0.3281 and 0.4895, respectively.

Genetic structure of *A. villosum* populations

Genetic structure information of *A. villosum* populations analyzed by Popgene 32 was shown in Table 5. Total genetic diversity (Ht) of the 7 populations was 0.3299, while the within population genetic diversity was 0.1819. Gene differentiation coefficient (Gst) was 0.4487, indicating that 55.13% of the genetic variation was existed within populations. The result was similar to that of molecular variance analysis (AMOVA), which showing 68.74% ($P=0.001$) genetic variation was in within populations while 31.26% ($P=0.001$) was between populations (Table 6). Additionally, the gene flow (Nm) among different populations was 0.6143.

Genetic distance and cluster analysis

Genetic distance is the main indicator to examine the degree of genetic differentiation and the relationship between groups. The genetic distances of 7 populations were between 0.0844 and 0.3347 (Table 7). Among them, the smallest genetic distance was between ZJD and TK population (0.0844), and the largest one was between XFC and YC population (0.3347). Mantel test carried out with NTSYS-pc 2.0 indicated that the genetic distance and geographical distance were not significantly correlated ($r = 0.02698$, $P = 0.5504$) (Fig. 3).

The UPGMA clustering map of populations based on genetic similarity coefficient was constructed using the data of Nei's genetic distance of seven populations (Fig. 4). *A. villosum* populations were divided into three groups at the similarity coefficient of 0.84. Three populations ZJD, TK and ZY formed one group. Three populations GY, MM and YC formed another group. One population XFC formed a single group. The results of PCoA based on the unbiased pair Φ_{st} matrix of Nei were consistent with UPGMA cluster analysis. And the first two principal components accounted for 48.04% of the total variation of ISSR markers indicating from PCoA (Fig. 5).

Discussion

Many DNA barcode reference libraries have been constructed for the purpose of a more rapid and accurate species identification [18, 19]. In this study, five DNA barcodes ITS2, *psbA-trnH*, ITS, *matK* and *rbcL* were amplified and sequenced from 141 individuals of seven *A. villosum* populations and finally 531 sequences were obtained. Thus the first local DNA barcode reference library of *A. villosum* in Guangdong Province was constructed. Fruits from the same genus plants, especially *A. xanthioides* Wall. ex Baker and *A. longiligulare* T.L. Wu are very morphologically similar to *A. villosum*'s and are also the genuine sources of Amomi Fructus. In addition, other adulterants of Amomi Fructus are sold in the market [20]. Huang etc have used seven DNA barcodes to identify the three genuine germplasms of Amomi Fructus [21]. An ideal DNA barcode should be easily retrievable and bi-directionally sequenced, and provide maximal discrimination among species [9]. From our results, the highest PCR amplification and

sequencing success rate of the five DNA barcodes for *A. villosum* was *rbcL*. For a more comprehensive assessment of discrimination power of DNA barcodes, it needs more investigations for discriminating power with adulterants of *A. villosum* included.

DNA markers especially ITS sequences are used in plant population genetic analysis [22]. In this study, we aligned the sequences within the DNA barcodes, but no variation sites of the five barcodes were found. Therefore, genetic diversity could not be analyzed by these DNA barcode markers. It might be caused by the narrow geographic collection of *A. villosum* populations [23]. Though intra-specific divergence could occur at a very high rate within geographically isolated populations [24], it seems that the geographic areas should be wide enough for the populations that could be analyzed of genetic diversity by DNA barcodes. And this is confirmed by other studies [25]. In fact, species identification and genetic diversity are two conflicting problems for the barcoding approach and these problems have to be dealt at the library construction stage [26]. Limited by sampling, however, how much variation is actually needed to separate species is not known with certainty [23]. It needs further research on this topic of field.

Then we analyzed the genetic diversity and genetic structure of *A. villosum* populations by ISSR marker through the whole genome. The results indicated that germplasm materials of *A. villosum* in Yangchun had relatively higher genetic diversity ($H = 0.3281$, $I = 0.3816$). Among the seven populations, population ZY had the highest genetic diversity, this is consistent with the fact that it had a diverse germplasm resources and made the research results more reliable. The G_{st} results (0.4487) indicated that there's obvious genetic differentiation among *A. villosum* populations [27] and more genetic variations existed in within populations. It was also confirmed by the AMOVA analysis. The gene flow value ($N_m = 0.6143$) revealed that genetic drift was the main cause for genetic variations among populations [28]. The weak gene flow of *A. villosum* populations is probably due to its pollination style and the habitat fragmentation. *A. villosum* is extremely difficult to self-pollinate caused by its morphological structure of flowers. Due to lacking of pollinating insects, *A. villosum* in Guangdong mainly relies on artificial pollination. The gene flow is consequently restricted to a small population. Another reason is that, habitat fragmentation of *A. villosum* in Yangchun is likely to reduce the exchange of spores between populations.

Amomi Fructus has been used in China for more than a thousand years and was mainly imported from abroad for a long history period. *A. villosum* has been cultivated in Yangchun for some 200 years and then was introduced into Guangxi, Yunnan and Fujian provinces in southern China [29]. The species with high genetic variation can resist the survival pressure caused by various environmental changes. *A. villosum* populations in Guangdong Province especially in the Daodi producing area of Amomi Fructus, Yangchun City, have high genetic diversity inferred from our study. And Yangchun has expanded the planting area of *A. villosum* in recent years. These are good news for *A. villosum* protection and cultivation. Though, considering the small populations and the increasingly fragmented habitat as well as the lower gene flow of *A. villosum*, we need to take continuous measures to protect the genetic resources of the species.

Conclusion

In the present study, a local DNA barcode reference library containing 531 sequences were constructed from 141 samples of seven *A. villosum* populations in Guangdong Province. On the other hand, the *A. villosum* populations have high genetic diversity but the gene flow was weak revealed by ISSR markers. The genetic relationship of each population was relatively close. More measures are needed to protect the genetic resources of *A. villosum* in Guangdong province, especially in Amomi Fructus's Daodi producing area, Yangchun city.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files except for some ISSR-PCR electrophoretic maps. They are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Lu Gong designed the experiment, analyzed the results and revised the manuscript. Danchun Zhang completed the experiment, data analysis and the draft manuscript. Xiaoxia Ding helped with sample collections and the experiment. Wan Guan helped do data analysis. Xiaohui Qiu made contributions to the conception of this work. Zhihai Huang made the conception and gained the supporting funds of this work.

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Tables

Table 1 Sampling information of *A. villosum* populations in Guangdong Province

No.	Pop.	Serial No.	longitude /N	Latitude /E	samples size
1	ZhongjiaoDong	ZJD	112°04' 01"	22°24' 16"	33
2	Tankui Village	TK	112°04' 30"	22°24' 22"	16
3	G325 National Roadside,	ZY	112°03' 59"	22°24' 26"	25
4	XingfuVillage,	XFC	112°00' 41"	22°21' 54"	18
5	National Geopark,	GY	111°49' 51"	22°35' 19"	19
6	Dianbai District, Maoming	MM	111°12' 21"	21°45' 52"	10
7	Datong Village	YC	111°58' 48"	22°23' 25"	20

Table 2 Efficiency of PCR and sequence characterization of DNA barcodes

	numbers of sequences	PCR amplification success rate (%)	sequencing success rate (%)	average sequence length /bp	GC content /%	conservative sites
ITS2	134	95.74	95.04	229	59.83	229
<i>psbA-trnH</i>	76	57.45	53.90	667	28.49	667
ITS	139	99.29	98.58	653	56.20	653
<i>matK</i>	41	29.08	29.08	787	29.22	787
<i>rbcL</i>	141	100.00	100.00	729	41.84	729

Table 3 ISSR banding patterns of seven *A. villosum* populations.

primers	sequences	Tm(°C)	amplification bands	polymorphic bands	PPB(%)
UBC808	(AG) ₈ C	52.00	11	11	100.00
UBC817	(CA) ₈ A	49.24	11	10	90.91
UBC825	(AC) ₈ T	52.00	12	12	100.00
UBC840	(GA) ₈ YT	52.74	12	10	83.33
UBC866	(CTC) ₆	56.60	6	4	66.67
UBC889	DBD-(AC) ₇	46.83	14	9	64.29
sum	—	—	66	56	84.85
mean	—	—	11	9.33	84.82

Table 4 Genetic diversity of *A. villosum* populations based on ISSR.

	NO.	Na	Ne	H	I	PPB(%)
ZJD	9	1.5303	1.3492	0.1998	0.2954	53.03
TK	4	1.2879	1.1848	0.1117	0.1658	28.79
ZY	7	1.7121	1.4240	0.2536	0.3816	71.21
XFC	5	1.4394	1.2685	0.1624	0.2434	43.94
GY	5	1.4848	1.3516	0.1998	0.2897	48.48
MM	3	1.4901	1.3273	0.1818	0.2604	40.91
YC	5	1.4394	1.2753	0.1648	0.2460	43.94
mean	7	1.4834	1.3115	0.1820	0.2689	47.19
At species level	38	1.9394	1.5662	0.3281	0.4895	

Table 5 Genetic structure of *A. villosum* populations based on ISSR

samples size	genetic diversity of the total populations (Ht)	genetic diversity within populations (Hs)	gene differentiation coefficient (Gst)	gene flow (Nm)
38	0.3299	0.1819	0.4487	0.6143
standard deviation	±0.0272	±0.0102	—	—

Table 6 AMOVA analysis of *A. villosum* populations based on ISSR

source of variation	degree of freedom /df	mean square deviation /SS	mean square value /MS	variance component	variance component percentage (%)	P
among populations	6	167.835	27.972	3.718	31.26	0.001
within populations	37	253.455	8.176	8.176	68.74	0.001
sum	43	421.289	—	11.894	100	—

Table 7 Nei's genetic distance of *A. villosum* populations

	ZJD	TK	ZY	XFC	GY	MM	YC
ZJD	0.0000						
TK	0.0844	0.0000					
ZY	0.0973	0.1247	0.0000				
XFC	0.2347	0.3317	0.1957	0.0000			
GY	0.2260	0.2697	0.1946	0.1463	0.0000		
MM	0.2785	0.2827	0.2218	0.2342	0.1132	0.0000	
YC	0.2697	0.2719	0.2176	0.3347	0.2003	0.1382	0.0000

Figures

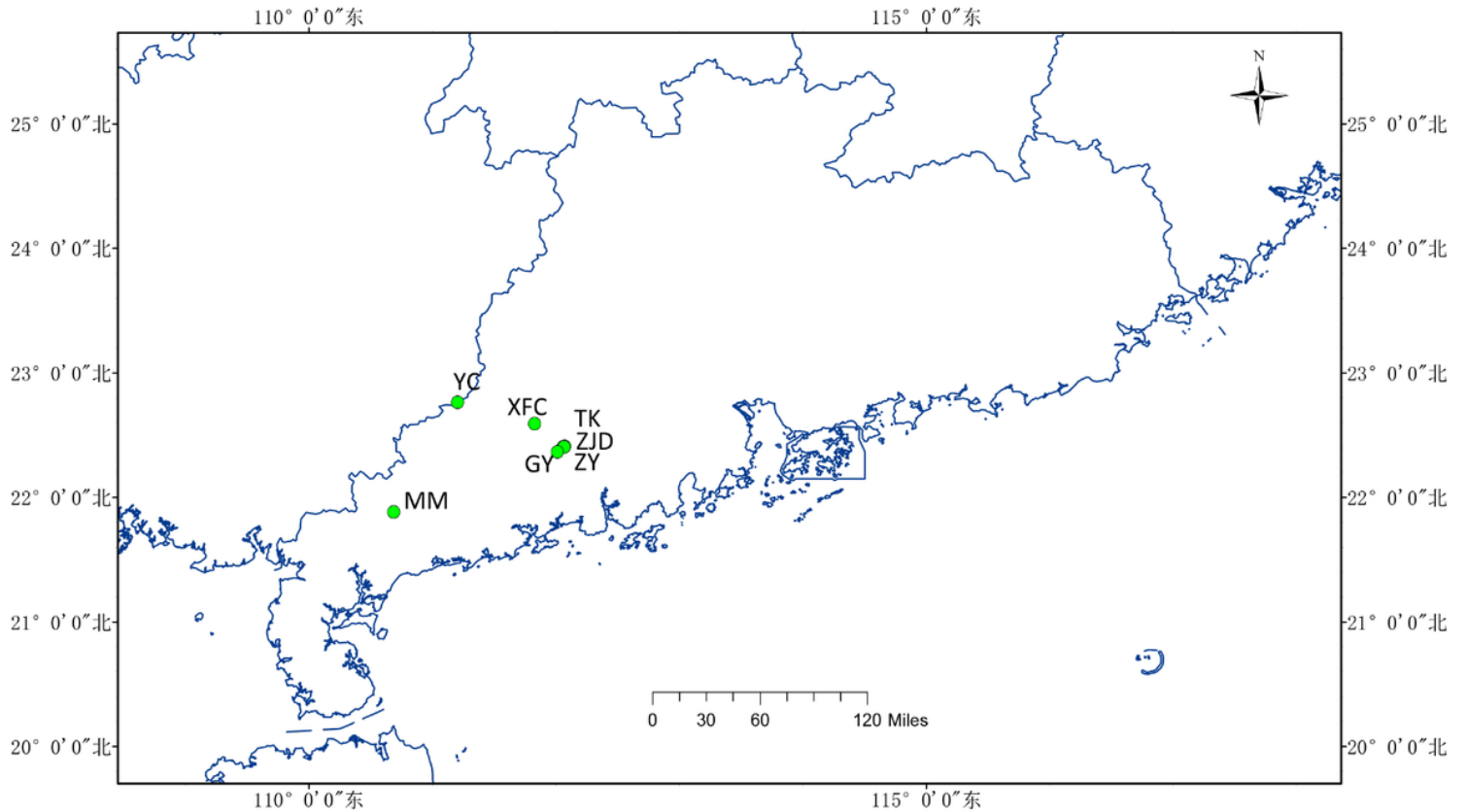


Figure 1

Geographical distribution of collected *A. villosum* populations Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

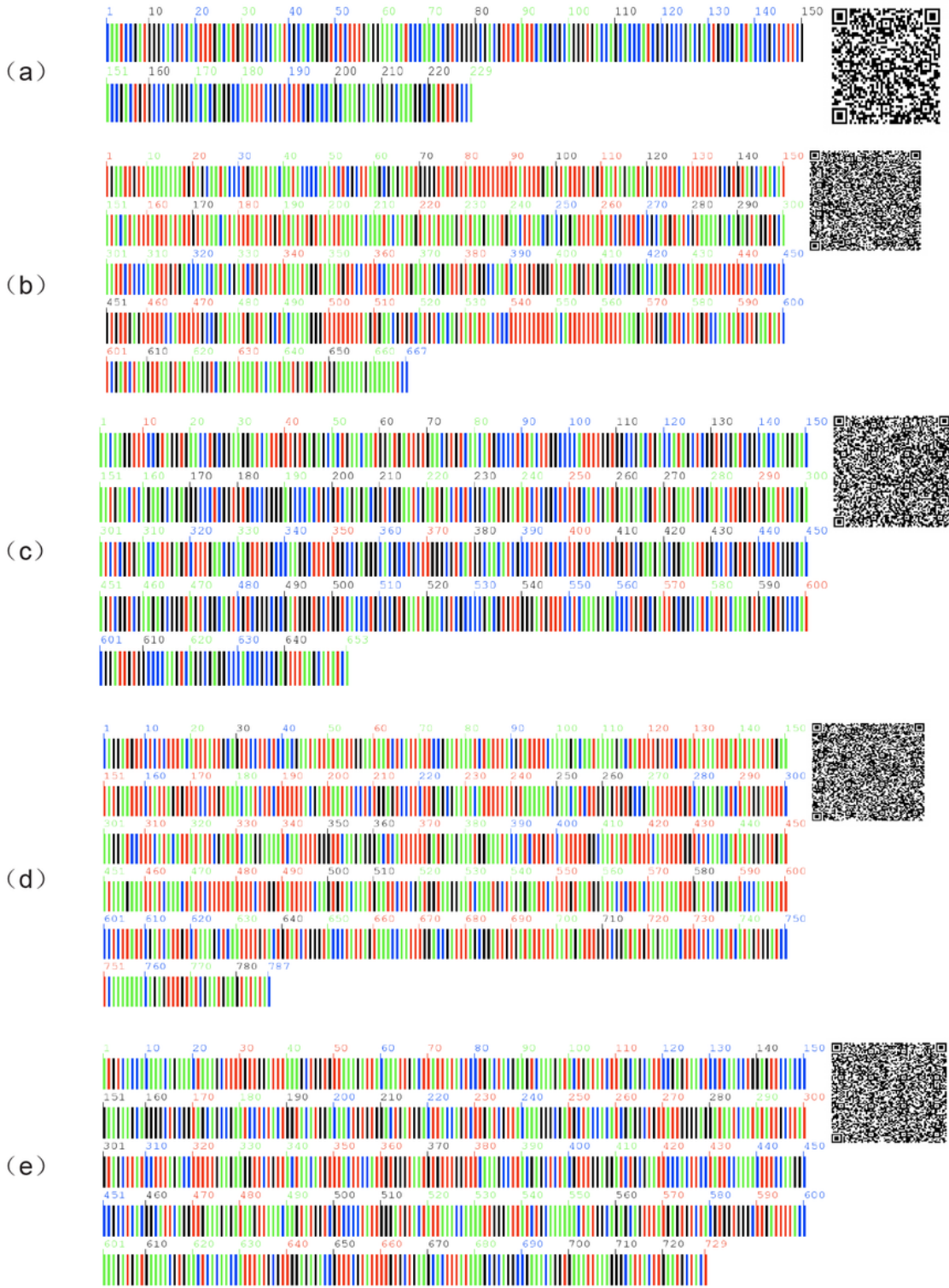


Figure 2

DNA barcode sequences of *A. villosum*.

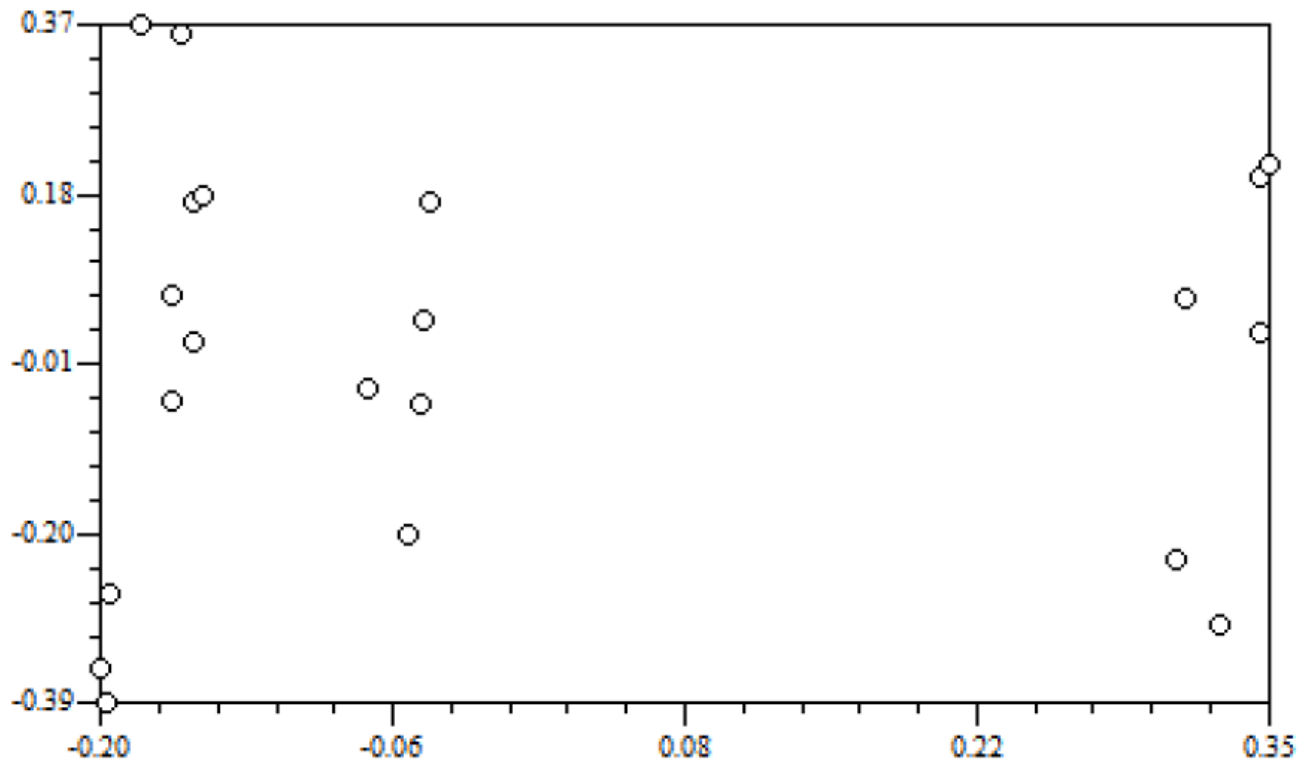


Figure 3

Correlation of geographic distance and genetic distance

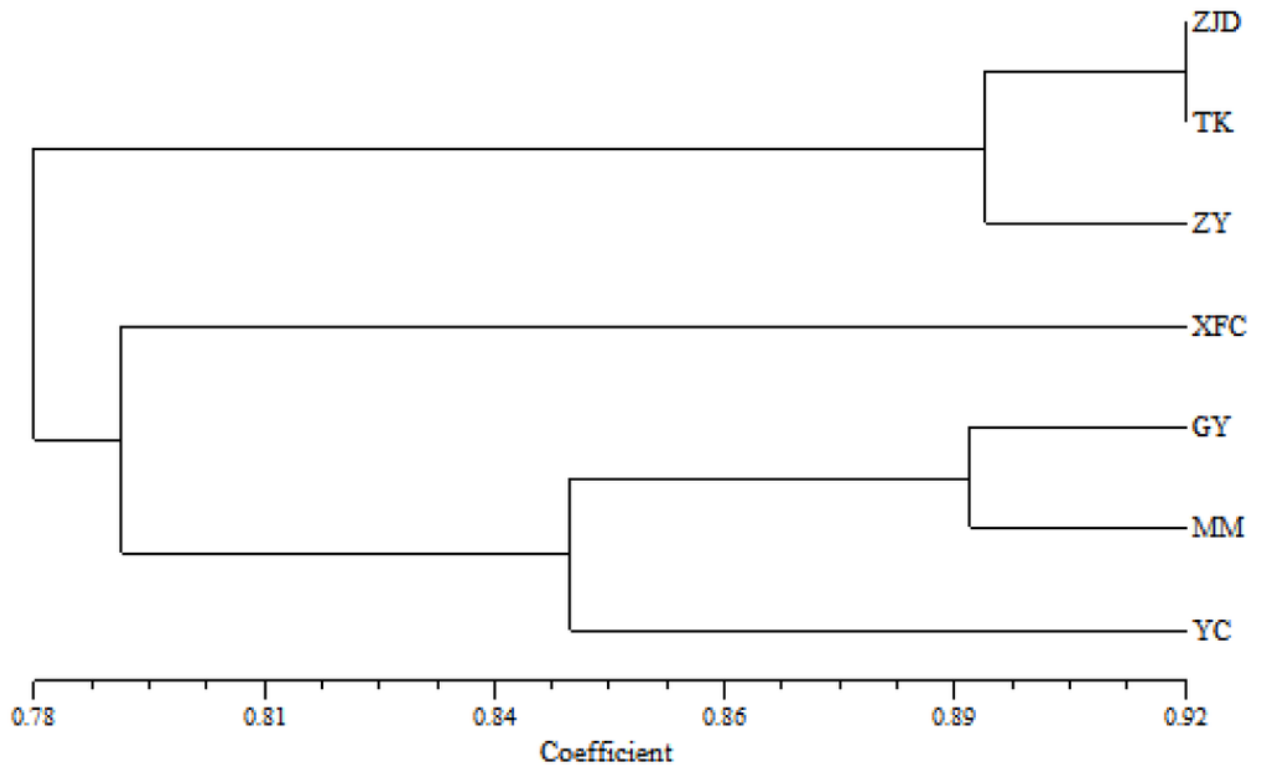


Figure 4

UPGMA clustering map of *A. villosum* populations

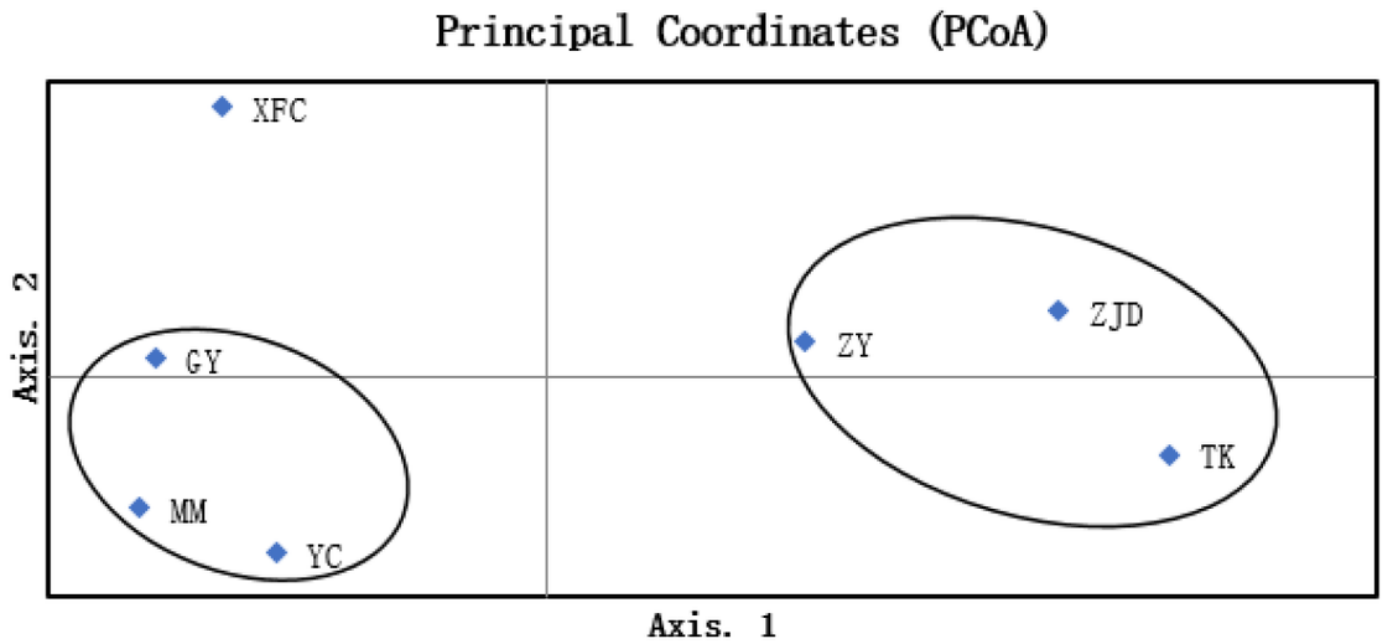


Figure 5

PCoA map of *A. villosum* populations (coord. 1 = 48.04% and coord. 2 = 26.74%)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supplementarymaterials.docx](#)