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Intraspecific variation of *Achyranthes bidentata* (Amaranthaceae) in the geo-authentic product area based on internal transcribed spacer sequences of ribosomal DNA

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Abstract

Achyranthes bidentata Blume belongs to perennial herbs (Amaranthaceae) and is widely distributed in India, Java, China, and Japan. The population of this plant is rapidly decreasing due to overexploitation for its important medicinal value. In this study, the genetic diversity of *A. bidentata* was analyzed using an internal transcribed spacer of ribosomal DNA from 58 individuals in the geo-authentic product area. A survey of ribosomal DNA variation revealed relatively low genetic diversity (h_T = 0.703) and significant genetic differentiation (Φ_{ST} = 0.730). Nucleotide diversity (π) among 8 populations ranged from 0 to 4.04×10^{-3} and haplotype diversity (h) varied between 0 and 0.822. Significant deviation from Hardy–Weinberg expectation ($P \leq 0.001$) was found in the species due to its high homozygosity. The population sampled from Jigong Mountain had the highest nucleotide and haplotype diversity.The isolation-by-distance for ribosomal DNA was detected in plants from the geo-authentic product area was not significant (r = 0.236, P = 0.134), and two major geographic groups were distinguished. Increasing human activity and overexploitation are likely to be the reasons for the observed high differentiation and relatively low genetic diversity. Based on the results of this study, the populations from Jiulian Mountain and Jigong Mountain, which had greater genetic diversity than the other sampled populations, should be the best candidates for *ex situ* conservation.

Keywords *Achyranthes bidentata*, genetic diversity, genetic differentiation, geo-authentic product area.
Abbreviations: internal transcribed spacer (ITS), total genetic diversity (h_T), genetic differentiation among populations (Φ_{ST}), gene flow among populations (*Nm*), observed heterozygosity (Ho), Hardy–Weinberg equilibrium (HWE), isolation by distance (IBD), ribosomal DNA (rDNA), Analyses of molecular variance (AMOVA), Unweighted Pair Group Method with Arithmetic Mean (UPGMA), Nucleotide diversity (π), haplotype diversity (h), Jigong Mountain (JG), Lingshan (LS), Huaiyuan (HY), Dacheng Mountain (DC), Longyuwan (LY), Laojieling (LJ), Jiulian Mountain (JL), Wanxian Mountain (WX).

Introduction

Achyranthes bidentata Blume is a perennial herb belonging to Amaranthaceae which is recognized as an important Chinese herbal medicine. It is widely distributed in India, Java, China, and Japan (Yang et al., 2012). In China, *A. bidentata* mainly grows in the majority of warm temperate and subtropical regions at elevations of 200-1,750 m. The species can reach a height of 1.2 m, which has erect stems with four prisms. Branches and simple leaves are opposite each other and the stem node is slightly swollen. Spikes are terminal or axillary, with lengths that reach up to 10 cm, and two thorn-like bracteoles persist in mature seeds. *A. bidentata* is in flower from August to September, and the seeds ripen from September to October. In addition, the flowers fold downward and close to the peduncle after florescence, and oblong utricles are wrapped in calyx (Editorial Board of the Flora of China, 1979). As an important Chinese medicinal herb, *A. bidentata* is used to treat cough, renal dropsy, fistula, scrofula, skin rash, nasal infection, chronic malaria, impotence, fever, asthma, amennorrhoea, piles, abdominal cramps and snake bites (Selvanayagam et al., 1994; Vetrichelvan and Jegadeesan, 2002). The therapeutic effects are attributed to the presence of important bioactive chemical compositions in the plant, including rutin, saponins, achyranthine, caffeic acid, oleanolic acid, inokosterone, ecdysterone, rubrosterone and physcion (Bishit et al., 1993; Nguyen et al., 1995; Chang et al., 1997). Previous studies have mainly focused on the pharmacology and biochemistry of *A. bidentata* (Meng and Li, 2001; Li, 2008), but the genetic diversity of the wild population has been rarely explored. In order to devise adequate conservation and management strategies for this species, it is important to characterize its genetic diversity and understand its population structure. Although *A. bidentata* is a widespread species in China, only samples from the geo-authentic product area of Henan Province have the best medicinal effect (Li, 2008). Because the species is not artificially cultivated, the wild population of this plant is rapidly dwindling due to overexploitation for the important medicinal value, especially in the geo-authentic product areas. In this study, the internal transcribed spacer (ITS) regions of ribosomal DNA were used to assess the extent and structure of genetic variation among and within populations of *A. bidentata* from the geo-authentic product area of Henan Province, China. As biallelic markers, the sequences of ITS regions have high levels of genetic variation and have been widely used to infer phylogenetic relationships and genetic diversity as well as unravel evolutionary pathways in a wide range of complexes in plants (Li et al., 2011; Alvarez and Wendel, 2003; Baldwin and Markos, 1998; Baldwin et al., 1995; Hershkovitz et al., 1999; Kelch and Baldwin, 2003;

Table 1. Variable sites of the aligned sequences of an Internal Transcribed Spacer (ITS) in 11 haplotypes of *Achyranthes bidentata*. Numbers 1/0 in sequences denote the presence/absence of length polymorphism, identified by superscript letters (a).

Nucletide Position	ITS										
	118	157	188	210	222	478	530	555	573	576	598
H1	C	C	G	G	T	C	C	G	C	1[a]	G
H2	C	C	G	G	C	C	C	G	C	1[a]	G
H3	T	C	G	G	C	C	C	G	C	1[a]	G
H4	C	C	G	G	C	C	C	A	C	1[a]	G
H5	C	C	G	G	T	T	C	G	C	1[a]	G
H6	C	C	A	G	C	C	T	G	T	1[a]	T
H7	C	C	A	G	C	C	C	G	C	1[a]	T
H8	C	C	A	T	C	C	C	G	T	0	T
H9	C	C	A	G	C	C	C	G	T	1[a]	T
H10	C	T	A	G	C	C	C	G	C	1[a]	T
H11	C	T	A	G	C	C	C	G	T	1[a]	T

Lee et al., 2002). The specific objectives were (i) to examine intraspecific genetic variation of *A. bidentata* in the geo-authentic product area; and (ii) to examine its genetic structure through DNA analysis.

Results

One ribosomal DNA (ITS) region was sequenced in *A. bidentata* from 58 individuals, 8 populations. The aligned sequences were 622 bp in length and contained ten nucleotide substitutions and one indel of 2 bp in length (Table 1). Based on these polymorphisms, eleven cpDNA haplotypes (H1–H11) were identified among all of the samples surveyed (Table 1). The sequences of eleven ITS haplotypes have been deposited in the GenBank database under accession numbers JQ621845–JQ621855. Among the eleven haplotypes detected, the most widespread haplotype was H1 (in 6 of 8 populations). The geographical distribution of haplotypes H1–H11 and their occurrence at each locality are shown in Figure 1. Six of the 8 populations were polymorphic (JG, LS, HY, DC, JL and WX), whereas the other two populations exhibited only one haplotype. The haplotype diversity and nucleotide diversity for *A. bidentata* were $h_T = 0.703$ and $\pi_T = 3.65\times10^{-3}$, respectively. Nucleotide diversity (π) among 8 populations ranged from 0 to 4.04×10^{-3} and haplotype diversity (h) varied between 0 and 0.822. The highest nucleotide diversity and haplotype diversity was found in population JG, and the lowest nucleotide diversity and haplotype diversity were found in populations LY and LJ, respectively (Table 2). The observed and expected heterozygosity among 8 populations ranged from 0 to 0.75, and from 0 to 0.74, respectively (Table 3). The highest observed heterozygosity were found in Population JL (H_o = 0.75). The observed heterozygosity of five populations (HY, DC, LY, LJ and WX) was 0. Three populations (JG, HY and DC) deviated significantly from the Hardy–Weinberg equilibrium (HWE) ($P \le 0.05$), which was due to an excess of homozygotes. The observed heterozygosity (H_o = 0.14) at the whole level was also significantly deviated from HWE due to the excess of in the number of homozygotes ($P \le 0.001$).

The rDNA data demonstrated significant population differentiation within *A. bidentata* (Φ_{ST} = 0.730) and the gene

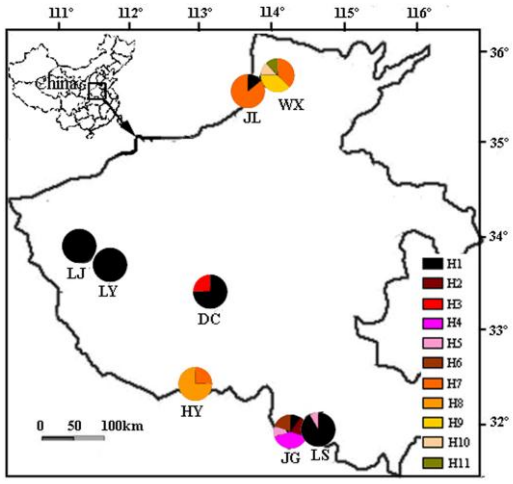

Fig 1. Sampling localities of 8 populations of *Achyranthes bidentata* and the geographic distribution of eleven cpDNA haplotypes (H1-11) detected. The population codes are the same as in Table 2.

flow among populations was extremely low (Nm = 0.11). Two groups were identified by neighbor joining tree of the population, whereby populations JL, WX and HY had close phylogenetic relations, JG, DC, LY, LJ and LS were also closely related populations (Fig. 2). The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree of haplotypes revealed that haplotype H1-H5 formed a monophyletic group and H6-H11 formed another monophyletic group (Fig. 3). In addition, analysis of molecular variance (AMOVA) revealed that 72.97% of the total rDNA variance was distributed among all populations and 27.03% of the variance was apportioned within populations (Table 4). No significant isolation-by-distance (IBD) for rDNA was detected in the geo-authentic product area (r = 0.236, P = 0.134). Tajima's D and Fu and Li's D* statistics for deviation from neutrality were non-significant for the entire geo-authentic product area ($P > 0.05$). Moreover, the observed mismatch distributions of haplotypes from the entire geo-authentic product area did not differ

Table 2. Details of population locations, sample size, and rDNA variation of *Achyranthes bidentata* sampled in Henan. N: number of sample individuals; *h*: haplotype diversity, π: nucleotide diversity.

Population no. and code	Locations	Lat.(N)/ Long.(E)	N	Haplotype	$\pi \times 10^{-3}$	h
1.JG	Jigong Mountain, Xinyang	31.83/114.07	5	1, 2, 4, 5, 6	4.04	0.822
2.LS	Lingshan, Xinyang	31.91/114.24	6	1, 5	0.27	0.167
3.HY	Huaiyuan, Nanyang	32.41/113.29	8	7, 8	1.29	0.400
4.DC	Dacheng Mountain, Nanyang	33.25/113.02	8	1, 3	1.29	0.400
5.LY	Longyuwan, Luoyang	33.70/111.76	8	1	0.00	0.000
6.LJ	Laojieling, Nanyang	33.63/111.77	8	1	0.00	0.000
7.JL	Jiulian Mountain, Xinxiang	35.54/113.61	8	7, 9, 10, 11	1.50	0.733
8.WX	Wanxian Mountain, Xinxiang	35.73/113.63	7	1, 7	1.27	0.264
product area mean					3.65	0.703

significantly from mismatches expected under models of constant population size (r = 0.116).

Discussion

Genetic diversity

In general, geographic distribution, breeding system and size of population all affect genetic diversity in plant species (Hamrick and Godt, 1989). Species that display out-crossing and mixed breeding typically have higher diversity than species that exhibit self-crossing. In addition, widespread species usually have higher genetic diversity than limited distributions, and a large population size is usually associated with a high genetic diversity (Hamrick and Godt, 1989, 1996; Hamrick et al., 1992; Nybom, 2004). As a dominant species in this region, *A. bidentata* is an out-crossing, animal-pollinated and widespread species (Li, 2008) that would be expected to have a relatively high genetic diversity. However, unexpectedly, the species-wide level of haplotype diversity in *A. bidentata* (h_T = 0.703) was lower than that of other seed plants in China based on the ITS, such as *Tacca chantrieri* (h_T = 0.732, Zhao and Zhang, 2011) and *Eriophyton wallichii* (h_T = 0.908, Wang et al., 2011). We also found that the species significantly deviated from HWE, which was due to an excess of homozygotes, Human activity has increased in this region in recent years, which may have disturbed the insect pollination and thereby improved the inbreeding coefficient and lowered the genetic diversity. In addition, the over-collection of these plants for medicinal purposes could have caused a sharp decline in the quantity of plants and the distribution area, which may have also accelerated the loss of genetic diversity. Among the populations investigated, the population JG (h = 0.822, π = 4.04×10^{-3}) and JL (h = 0.733, π = 1.50×10^{-3}) had the highest nucleotide and haplotype diversity compared to the others, and these two populations occupied the most haplotypes (in 9 of 11 haplotypes). In general, the population that has high gene diversity should be selected as the unit for *ex situ* conservation. Therefore, the JG and JL populations, which had greater genetic diversity, are the best candidates for *ex situ* conservation.

Genetic structure

The rDNA data demonstrated significant population differentiation within *A. bidentata* (Φ_{ST} = 0.730), which was due to the limited gene flow (Nm = 0.11) among populations. An analysis of the degree of genetic isolation with increasing geographic distance (IBD) was not statistically significant based on rDNA analysis (r = 0.116; P > 0.05), suggesting that there is no relation between genetic distance and geographic distance. In general, restricted gene flow, drift, and inbreeding tend to increase genetic differentiation among populations (Jacquemyn et al., 2004).

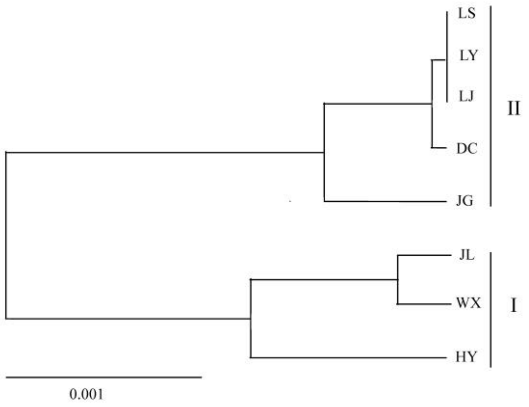

Fig 2. Neighbor-joining (NJ) clustering of 8 populations of *Achyranthes bidentata* based on their pairwise genetic distances in rDNA as determined from net average (D_A) nucleotide divergences (Tamura and Nei 1993). The population numbers are identified in Table 1. Group I: populations HY, JL and WX; Group II: populations JG, DC, LY, LJ, and LS.

The mature seeds of *A. bidentata* are small with thorn-like bracteoles (Li, 2008), and seeds with a similar structure are usually dispersed by animals. However, the increase in human activity in this region has hindered seed dispersal. Tajima's D and Fu and Li's D^* statistics together with the multimodal mismatch distribution also suggested that significant population expansion did not occur for *A. bidentata*, which is consistent with the limited dispersal hypothesis. Limited pollen flow among populations was considered to be another explanation for the high population differentiation, and the excess number of homozygotes also confirmed this hypothesis. The partitioning of genetic variability had a significant geographic component and two major groups were identified (Fig. 2), including I (HY, WX and JL) and II (JG, DC, LY, LJ, and LS). Phylogenetic analyses of 11 rDNA haplotypes also supported this subdivision (Fig. 3). The UPGMA tree of haplotypes revealed that H1–5 formed a monophyletic group and H6–11 formed another monophyletic group. The H7–11 haplotypes were grouped in HY, WX and JL; however, H6 was grouped into group I rather than group II, which was only found in one individual. The H1–5 haplotypes were identified in WX, JG, DC, LY, LJ, and LS. In population WX, the H1 haplotype was only identified in one individual. Therefore, the limited gene flow is likely to have prevented recent gene flow among regions. In addition, increasing human activity and overexploitation is likely to result in habitat fragmentation.

Table 3. Results of each population and the entire geo-authentic product area values of expected heterozygosity and observed heterozygosity.

Population no. and code	No. of alleles	Expected heterozygosity	Observed heterozygosity
1.JG	5	0.74	0.20*
2.LS	2	0.15	0.16
3.HY	2	0.38	0.00*
4.DC	2	0.38	0.00*
5.LY	1	0.00	0.00
6.LJ	1	0.00	0.00
7.JL	4	0.69	0.75
8.WX	2	0.24	0.00
product area mean	11	0.70	0.14**

Statistically significant deviation from Hardy–Weinberg expectation is indicated by * ($P \leq 0.05$), ** ($P \leq 0.001$).

Table 4. Analysis of molecular variance (AMOVA) for populations of *Achyranthes bidentata* based on rDNA data (d.f., degree of freedom; SSD, sum of squares); **$P \leq 0.001$.

Source of variation	d.f.	SSD	Variance component	Percentage of variation
Among populations	7	94.296	0.90874	72.97**
Within populations	108	36.360	0.33666	27.03**

Based on these findings, we suggest that recent range fragmentation was the main reason for the isolation of the two groups.

Materials and methods

Population sampling

Individuals used in this study of *A. bidentata* were randomly chosen for sampling from each of eight wild populations in the geo-authentic product area of Henan Province, China (Table 1. Fig. 1). All individuals at least 10 m apart were sampled for each population in order to increase the likelihood of sampling inter-individual variation within each population. Young leaves were collected and dried in silica gel and stored at -20 °C for DNA extraction.

DNA isolation, PCR amplification and DNA sequencing

Total DNA was extracted from roughly 30 mg of dried leaf tissue using a Plant Genomic DNA Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. DNA concentrations were determined (i) on ethidium bromide-stained 1% agarose gels by comparison with known amounts of λ DNA, and (ii) by spectrophotometry. Working stocks of DNA were then prepared based on both estimates and stored in 0.1 × TE buffer. After preliminary screening, we chose ITS for the full survey. This was amplified through polymerase chain reactions (PCRs) using primers ITS4-ITS5 (White et al., 1990). The PCR reactions were carried out in a PTC-200 thermal cycler (MJ Research, Watertown, MA, USA) using a total volume of 30 μL containing 30 ng of genomic DNA, 0.2 mM of each dNTP, 0.3 μM of each primer, 3μL *Taq* buffer and 1 unit *Ta*q polymerase (Takara, Dalian, Liaoning, China). The PCR program was as follows: 4 min initial denaturation at 94 °C, followed by 35 cycles of 40 s denaturation at 94 °C, 45 s annealing at 50 °C, and 1 min 30 s elongation at 72 °C. This was followed by a final extension at 72 °C for 8 min. The PCR products were purified with an E.Z.N.A® Gel Extraction Kit (Omega Bio-Tek, Winooski, VT, USA) and then sequenced on an ABI 3730 DNA Sequence Analyzer at Shanghai Invitrogen Biotechnology Co., Ltd (Shanghai, China).

Data Analysis

The DNA sequences were edited manually based on the chromatograms and aligned by CLUSTAL_X (version 1.81; Thompson et al., 1997). After the alignment, one indel identified was coded as substitutions following Caicedo and Schaal (2004). Because *A. bidentata* is diploid (2n = 42), we counted the two alleles of each sampled individual and treated a site as polymorphic if there was a "double peak" in the chromatogram. Haplotypes of a nuclear gene were inferred using "haplotype subtraction" (Zhou et al., 2007; Clark, 1990; Olsen and Schaal, 1999). Haplotype diversity (h) and nucleotide diversity (π) were calculated for each population (h_S, π_S) and at the species level (h_T, π_T) using DNASP (version 4.0; Rozas et al., 2003). Gene flow (*Nm*) (Hudson et al., 1992) among populations was also calculated by DNASP. The expected heterozygosity, observed heterozygosity, and Hardy-Weinberg equilibrium (HWE) of each population and the entire geo-authentic product area were determined using GENEPOP version 4.0.10 (http://genepop.curtin.edu.au/) (Raymond and Rousset, 1995). AMOVAs were used to calculate genetic variance components and their significance levels among populations

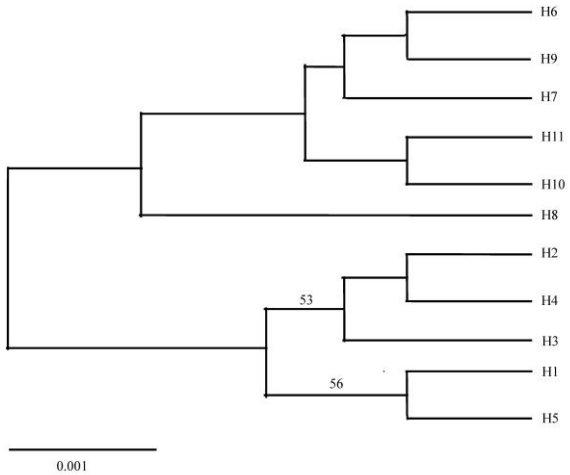

Fig 3. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering of eleven nuclear gene haplotypes sequences of *A. bidentata*. The H1–5 haplotypes formed a monophyletic group and the H6–11 haplotypes formed another monophyletic group. The bootstrap confidence values (%) are indicated on the branches.

and within populations by ARLEQUIN (version 3.1; Excoffier et al., 2005). Genetic relationships among 8 populations of *A. bidentata* were illustrated using the procedures NEIGHBOR and CONSENSE of the program PHYLIP (version 3.63; Felsenstein, 2004). Phylogenetic relationships between rDNA haplotypes of *A. bidentata* were assessed under the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in PAUP* (version 4.0 beta10; Swofford, 2002). To determine if the obtained rDNA sequences satisfied the assumption of neutrality, we calculated Tajima's *D* (Tajima, 1989) and Fu and Li's *D** (Fu and Li, 1993) for the entire geo-authentic product area using DNASP. The statistical significance of *D* and *D** was estimated with coalescent simulations as implemented in this program. In general, significant negative departures of these statistics from zero indicate deviation from neutrality, but may also represent evidence of recent demographic expansions or population bottlenecks when markers are otherwise assumed to be independent of selection (Tajima, 1989; Fu, 1997). To further infer demographic processes, we explicitly tested the null hypotheses of constant population size in DNASP by comparing the observed and expected distributions of pairwise sequence differences (mismatch distributions). For rDNA data, a test of isolation-by-distance (IBD) was performed by regressing values of Φ_{ST} against the geographic distance (*Km*) with the Mantel permutation procedure as implemented in IBD (Jensen et al., 2005; Isolation by distance, web service, http://ibdws.sdsu.edu/).

Conclusions

This analysis of rDNA variation in *A. bidentata* suggests a relatively lower genetic diversity than other species. The AMOVA analyses together with the genetic analyses of population strongly suggest a significant population subdivision. However, this subdivision did not correspond to geographic distance. Because of the interference of human activity, the species was found to have an excess of homozygotes, and significantly deviated from the HWE. The estimate of genetic diversity and population genetic structure of *A. bidentata* in the geo-authentic product area will help us in determining how to conserve this species. In this study, greater genetic diversity was found in populations JG and JL, which are the best candidates for *ex situ* conservation.

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