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Efficiency of ISSR markers in assessing genetic diversity and relationships in *Ardisia gigantifolia* germplasm

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Abstract: *Ardisia gigantifolia* is a medicinal plant that belongs to genus *Ardisia*. This plant has been an important traditional Chinese medicine in China for thousands of years. Now, with the development of pharmacological research, *A. gigantifolia* is not only used as a clinical medicine, but also plays a role in diet and health aspects. The pharmacological validity of its medicinal value has extended in an enlarged market. At present, the wild *A. gigantifolia* in our country is rare, and the cultivars become the new important source. However, the quality and efficacy of cultivars often suffer from intraspecific variation, unstable quality and other issues. Moreover, the cultivars were also affected by origin, age and environmental conditions. In order to efficiently utilize and conserve this medicinal plant resource, ISSR analysis was employed to reveal genetic diversity of 36 *A. gigantifolia* germplasm which were collected from different places of Guangxi Zhuang Autonomous Region in China. Data were analyzed by POPGENE32, and a cluster diagram was presented by UPGMA. The results indicated that fourteen ISSR primers generated a total of 136 bands among which 112 (82.35%) were polymorphic bands. Nei's genetic diversity index (H) was 0.296 5, Shannon diversity index (I) was 0.441 7, and the coefficient of gene differentiation (Gst) was 0.855 8. The genetic similarity coefficient among the populations ranged from 0.667 8 to 0.838 2 in an average of 0.739 1. Based on the clustering analysis, all accessions were clustered into five groups with UPGMA method. Most of the accessions from the same or adjacent regions were clustered into the same group or subgroups. A few accessions, however, were greatly different from the majority of all accessions. The results suggested that ISSR marker was an effective tool for the study of genetic variation in Chinese natural *A. gigantifolia* germplasm resources. These results can also be used for germplasm characterization and plant breeding.

Key words: *Ardisia gigantifolia*, molecular marker, ISSR analysis, germplasm resources, genetic diversity

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走马胎种质资源遗传多样性的 ISSR 分析

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摘 要: 走马胎(*Ardisia gigantifolia*)是紫金牛科(Myrsinaceae)紫金牛属(*Ardisia*)多年生小灌木植物。走马胎作为我国传统中药材,已有多年的药用历史。目前,走马胎不再局限于临床药用,在食疗和保健方面的开发利用崭露头角,大大扩展了其应用范围。随着走马胎市场需求量的增大,野生走马胎植物被过度采挖,导致野生走马胎资源几乎枯竭。人工栽培走马胎逐渐成为供应药用市场的主力军,但是人工栽培走马胎种质、种子来源混杂,常会造成质量和疗效的不稳定,而利用分子标记技术可以从分子水平上对走马胎进行种质的区分和评价。该研究利用 ISSR 分子标记技术,对来自广西地区的 36 份走马胎种质资源进行了遗传多样性分析,采用 POPGEN32 软件进行数据分析,用 UPGMA 软件绘制聚类图。结果表明:14 条 ISSR 引物共检测到 136 个清晰的扩增位点,多态性位点 112 个,多态位点百分率为 82.35%;Nei's 基因多样性指数(*H*)为0.296 5,Shannon 多样性指数(*I*)为0.441 7,基因分化系数(*G*st)为0.855 8。个体间的遗传相似系数为0.667 8~0.838 2,平均为0.739 1。基于聚类分析可知,所有的个体被划分为 5 类,其中绝大多数来自相同或者邻近地区的个体严格按照地理位置聚为相同的一类或者亚类,只有少数个体在归类上与地理位置相悖。研究证明 ISSR 分子标记技术在评价走马胎种质资源亲缘关系和遗传变异等方面有很好的适用。该研究结果为该药用植物的种质资源评估和引种栽培提供了科学依据。

关键词: 走马胎, 分子标记, ISSR 分子检测, 种质资源, 遗传多样性

Ardisia is the largest genus in the family of the Myrsinaceae, approximately consisting of 500 species of evergreen shrubs and trees throughout the subtropical and tropical regions of the world (Chen & Pipoly, 1996). *Ardisia* species have been used as sources for both food and folk medicine. *Ardisia gigantifolia*, is mainly distributed in Guangxi Zhuang Autonanous Region, Guangdong, Fujian and Jiangxi provinces, China (Feng et al, 2011). This plant has been used as an important traditional Chinese medicine to treat rheumatoid arthritis and inflammatory diseases in China for thousands of years as evident by ancient records (Mu et al, 2001). *A. gigantifolia* is also used for treatment of bruises sprains, blood stasis, tumors and chronic ulcers diseases. Recently, diverse types of compounds have been isolated from this medicinal plant, such as saponins, coumarins, and quinones (Kobayashi & De Mejía, 2005). In addition, very interesting bioactivities, such as anti-tumor, anti-inflammation, antivirus, anti-HIV, and anti-oxidation properties have been described for compounds isolated from this species (Kobayashi & De Mejía, 2005). A literature survey showed that *A. gigantifolia* contained dimeric 1, 4-benzoquinone derivatives that are rarely found in nature (Liu et al, 2009). However, with the market demand increasing and destruction of wild resources intensifying, *A. gigantifolia* is being destroyed at an alarming rate, and has been listed as the rare medicinal plant (Mao et al, 2010). Investigation of germplasm resources and evaluation of genetic diversity of *A. gigantifolia* will help ascertain the pre sent condition of this medicinal plant and thus offer practical advices for its further breeding, utilization and conservation.

Genetic diversity analysis has proven to be a useful strategy for revealing genetic backgrounds and relationships of germplasm resources. In the past decades, research on *A. gigantifolia* has been carried out. How ever, most of these studies focused on morphology, cytology and biochemistry, very little is known about the genetic diversity and genetic variation in *A. gigantifolia* (Gao et al, 2015; Tuo & Wang, 2012; Shu et al, 2011). Molecular markers based on polymerase chain reaction (PCR) are widely used since they require small amount of DNA and are effective and technically

simple. The more popular markers are random amplified polymorphic DNA (RAPD) (Li & Nelson, 2002), simple sequence repeat (SSR) (Qi et al, 2004), and inter-simple sequence repeat (ISSR) (Van der Nest et al, 2000). The principle of ISSR is similar to RAPD in that randomly sequence primers used. However, the ISSR primer sequences consist of a di-or tri-nucleotide simple sequence repeat amplifying the regions between two microsatellite repeats. As compared with RAPD, ISSR markers are more reproducible (Semagn et al, 2006) with polymorphic bands produced (Reddy et al, 2002). Since its establishment, ISSR has been widely used for analyses on genetic diversity, gene mapping and phylogenetic relationship in multiple species (Sun et al, 2014; Wei et al, 2014; Li et al, 2014; Li et al, 2015).

The identification and estimation of genetic diversity and relationships of elite varieties or promising varieties of *A. gigantifolia* is important because it provides the basic information for breeding programs and conservation and can ultimately have a direct effect on the production and quality of this medicinal plant. In this work, our aims were to construct the fingerprinting and assess the genetic diversity of *A. gigantifolia* germplasm resources from different places of Guangxi in China using ISSR markers. Our study will uncover valuable raw materials that provide crucial genetic diversity information for conservation and breeding of *A. gigantifolia*.

1　Materials and Methods

Though wild *A. gigantifolia* is distributed in Guangxi Zhuang Autonornous Region, Guangdong, Jiangxi and Fujian provinces, etc. in China, among which Guangxi is the main distribution region. Unfortunately, the wild *A. gigantifolia* is dispersedly distributed with very small number of individuals so that it is very difficult to find and collect. To evaluate the sampling strategies, we surveyed, collected, selected, conserved and documented 52 germplasm resources of *A. gigantifolia* mainly by phenotypic traits over a period of five years. According to geographic origins, all accessions of germplasm resources have been planted and conserved in the germplasm repository in Guangxi Institute of Botany, Guilin, China. For molecular assays, a total of 36 samples were originally and randomly collected from these plants. Fresh leaves in squaring period were randomly collected and desiccated in silica gel for molecular analyses. The details of the studied samples are summarized in Table 1.

Extraction of DNA was performed with 0.1 g starting material of leaves by using the Plant Genomic DNA Extraction kit (Tiangen Biotech Co., China), according to the manufacturer's instructions. The DNA concentration was estimated by standard spectrophotometric method at 260 and 280 nm UV lengths by Thermo Scientific Nanodrop 2 000 and the integrity by gel electrophoresis in a 0.8% agarose gel. DNA samples were then diluted to 30 ng · μL^{-1} work concentration.

A total of 100 ISSR primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (China), according to the public biotechnology website of University of British Columbia. These primers were then screened using a few DNA samples and the primers which yielded polymorphism bands were used initially for amplification to optimize the PCR conditions. Finally, fourteen of them yielded clearly, reproducible and relatively high polymorphism bands were used for ISSR analysis (Table 2). The PCR reaction was carried out in 25 μL of mixture containing 30 ng of genomic DNA, 2.0 mmol · L^{-1} $MgCl_2$, 0.2 mmol · L^{-1} dNTP, 10 × PCR buffer, 1 mmol · L^{-1} primer and 1 U Taq DNA polymerase (Sangon Biotech. Co., Ltd., Shanghai, China). The PCR cycling conditions were as follows: 94 ℃ for 3 min (initial denaturation), then followed by 39 cycles at 94 ℃ for 30 s (denaturation), annealing at optimal temperature for 45 s, 72 ℃ for 2 min (extension), with a final 7 min extension at 72 ℃ and then a cool down to 4 ℃. The amplified ISSR fragments along with DL2000 DNA marker (TaKaRa Co., Ltd., Dalian, China) were resolved in 1.5% agarose gels in TAE buffer at 110 V for 50 min. Gels with amplification fragments were visua lized and photographed under UV light.

The amplified DNA fragments were identified as present (1) or absent (0). The dataset was converted into a mathematical matrix used by the POPGENE32

Table 1 Description of the experimental materials

Sample code	Collecting time	Location	Altitude (m)	Pop. status and amount
A01	2012.10.24	Rongshui, Liuzhou	322	Wild, ≤5
A02	2012.10.26	Rongshui, Liuzhou	494	Wild, ≤5
A03	2012.10.28	Huanjiang, Hechi	494	Wild, ≤5
A04	2012.12.11	Jinxiu, Laibin	794	Wild, ≤5
A05	2012.12.12	Jinxiu, Laibin	576	Wild, ≤10
A06	2012.12.12	Jinxiu, Laibin	576	Wild, ≤10
A07	2012.12.31	Napo, Baise	445	Wild, ≥50
A08	2013.1.10	Ningming, Chongzuo	390	Wild, ≤5
A09	2013.1.16	Shangsi, Fangchenggang	500	Wild, ≤5
A10	2013.6.9	Napo, Baise	740	Wild, ≥50
A11	2013.6.9	Napo, Baise	659	Wild, ≤20
A12	2013.6.10	Napo, Baise	433	Wild, ≥50
A13	2013.6.11	Debao, Baise	—	Wild, ≤20
A14	2013.6.12	Debao, Baise	—	Wild, ≤20
A15	2013.6.12	Tiandeng, Chongzuo	—	Wild, ≤20
A16	2013.6.12	Tiandeng, Chongzuo	—	Wild, ≤20
A17	2013.6.12	Jingxi, Baise	—	Wild, ≤5
A18	2013.6.22	Gongcheng, Guilin	—	Cultivated
A19	2013.11.1	Lingui, Guilin	—	Cultivated
A20	2013.12.24	Jinxiu, Laibin	—	Wild, ≤5
A21	2013.12.25	Jinxiu, Laibin	987	Wild, ≤5
A22	2013.12.26	Jinxiu, Laibin	—	Cultivated
A23	2013.12.26	Jinxiu, Laibin	—	Cultivated
A24	2013.12.26	Jinxiu, Laibin	358	Wild, ≤20
A25	2013.12.26	Jinxiu, Laibin	459	Wild, ≤20
A26	2013.12.27	Jinxiu, Laibin	501	Wild, ≤20
A27	2013.12.27	Jinxiu, Laibin	674	Wild, ≤20
A28	2013.12.28	Jinxiu, Laibin	674	Wild, ≤20
A29	2013.12.27	Jinxiu, Laibin	486	Wild, ≤20
A30	2013.12.28	Jinxiu, Laibin	486	Wild, ≤5
A31	2013.12.29	Jinxiu, Laibin	870	Wild, ≤5
A32	2013.12.30	Jinxiu, Laibin	903	Wild, ≤5
A33	2014.5.30	Napo, Baise	1000	Wild, ≤20
A34	2014.5.28	Napo, Baise	400	Wild, ≤20
A35	2014.5.29	Napo, Baise	700	Wild, ≤20
A36	2014.5.30	Naliang, Fangchenggang	195	Wild, ≤20

software to perform statistical analyses, and calculate the index of genetic diversity, the observed total number of alleles (*Na*), effective allele number (*Ne*), percentage of polymorphic bands (*PPB*), Nei gene diversity (*He*, Nei, 1973), and Shannon's information index (*I*, Shannon & Weaver, 1949). A cluster dendrogram (UPGMA) was constructed to evaluate the genetic relationship for these accessions based on the average gene tic distances using the NTSYS software (Yeh et al, 1997; Rohlf, 2000).

2 Results and Analysis

The extent of genetic diversity among the thirty-six accessions of *A. gigantifolia*, a total of 100 ISSR primers were screened and fourteen primers yielded clearly, reproducible and relatively high polymorphism bands were selected for further ISSR analysis (Fig. 1). ISSR primers produced different numbers of DNA fragments, depending upon their simple sequence repeat motifs. The fourteen selected primers generated altogether 136 unambiguous and reproducible bands, of which 112 (82.35%) were polymorphic, the sizes ranging from 250 to 2 000 bp, the numbers of bands varied from eight to twelve, with an average of 9.7 bands per primer (Table 2).

The number of alleles is one of the most important genetic components for genetic diversification in populations. The percentage of polymorphicbands (PPB) for this species was 82.35% (Table 2). The mean observed number of alleles (*Na*) was 1.823 5, while the mean effective number of alleles (*Ne*) was 1.510 1. The Nei's gene diversity (*H*) was 0.296 5, and Shannon's indices (*I*) was 0.441 7. These results demonstrate that *A. gigantifolia* has a relatively high level of genetic diversity.

The total gene diversity (*Ht*) and the gene diversity within populations (*Hs*) were 0.274 1 and 0.039 5 respectively. According to $Gst = Ht - Hs/Ht$, all the samples of this species from different locations had a coefficient of genetic diversification (*Gst*) of 0.855 8.

Based on similarity coefficient among 36 samples, cluster analysis was carried out using UPGMA method and resulted in a phylogenetic dendrogram shown in Fig. 2. All the samples that could be classified into two

Table 2　Fourteen ISSR primer sequences and amplification results

Code of primer	Sequence of primer (5′-3′)	Number of amplified band	Number of polymorphic band
UBC827	$(AC)_8G$	12	11
UBC834	$(AG)_8YT$	12	9
UBC836	$(AG)_8YA$	9	9
UBC840	(GA)8YT	9	9
UBC845	(CT)8RG	11	8
UBC856	$(AC)_8YA$	11	8
UBC868	(GAA)6	8	6
UBC873	(GACA)4	8	7
UBC886	VDV(CT)7	8	5
UBC889	DBD(AC)7	9	8
UBC890	VHV(GT)7	9	9
UBC891	HVA(TG)	11	9
UBC899	CTAGGTGTTGGTCATTGTTCCA	9	6
UBC900	ACTTCCCCACAGGTTAACACA	10	8
Total	—	136	112
Average	—	9.7	8

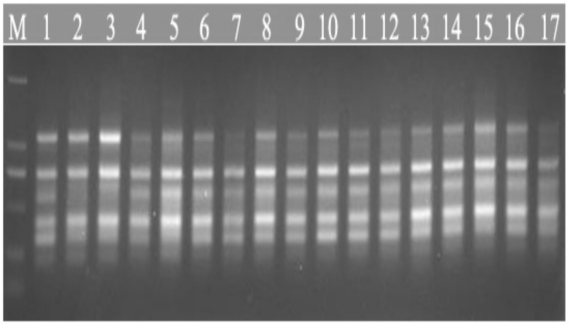

Fig. 1　Electrophoresis of *A. gigantifolia* DNA samples

big groups and then further divided into five sub-groups with a similarity coefficient value of 0.70. The clustering result of *A. gigantifolia* samples corresponded generally with the geographical distribution of their collections. However, there were still a few accessions could not be distinguished clearly. Generally, the result of PCoA was in accordance with that of genetic clustering analysis (Fig. 3). These findings clearly indicate a distinct differentiation between *A. gigantifolia* germplasms from various geographic origins.

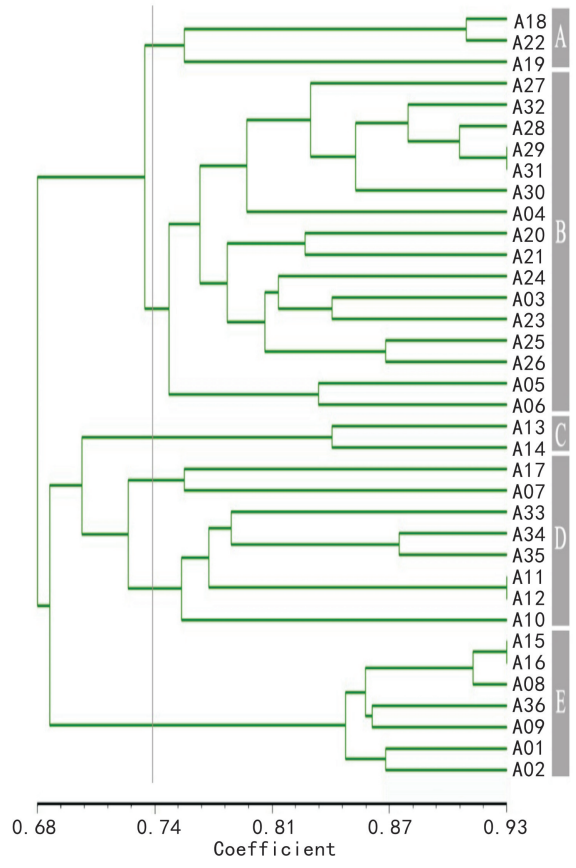

Fig. 2　UPGMA dendrogram generated from ISSR data of 36 *A. gigantifolia* accessions

3　Discussion and Conclusion

In recent years, a number of quantitative measures of genetic diversity have been proposed in the context of species conservation (Krajewski, 1994). The studies of the genetic diversity do not only help us to understand the present condition and appreciate genetic structure of our resources, but also help conserve biodiversity, as well as development and utilization of germplasms. In China, there are countless medicinal plants that have been widely used for treatment of many kinds of diseases. Nevertheless, with the increasingly serious environmental damage and excessive collection, many wild germplasm resources are facing destruction and are in danger of extinction. It would be an unprecedented dis-

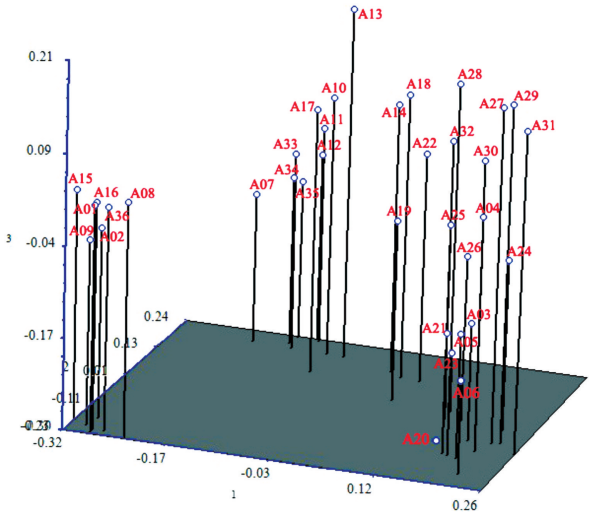

Fig. 3 Three-dimensional plot of the principal coordinate analysis (PCoA) of distance among 36 *A. gigantifolia* accessions

aster if medical cures were lost due to the extinction of these plants. Although *A. gigantifolia* had a long cultivating history as medicinal plant, there is little research on evaluating its germplasm diversity and how to construct a core collection. We investigated and evaluated the wild and cultivated *A. gigantifolia* in the past few years. Our results showed that the wild resources had been excessively excavated in the past decade and the wild resources reserves were not sufficient to meet the growing demands of medical utilization and chemical extraction industry. The cultivating area of *A. gigantifolia* has been increasing in recent years but still inadequate to meet the supply and demand in China. Some old problems are still unresolved and new problems are arising, such as decline in genetic diversity, yield and quality, etc. All these problems need to be resolved in the future. The development of a core collection will help cultivators create low-cost strategies to evaluate this medicinal plant germplasm.

There are different sampling strategies to construct core collection for germplasm resources. Cluster and principal coordinate analysis have been widely used as an important tool to group samples for constructing core collections (Zhang et al, 2004; Hintum, 1995) and grouping data based on molecular markers (Chabane & Valkoun, 2004). In the present study, we tested the usability of ISSR primers to investigate the level and distribution of genetic diversity in wild and cultivated populations of *A. gigantifolia* from Guangxi. We used fourteen out of the 100 ISSR primers tested, and 112 polymorphic bands in a total of 136 bands (*PPB* = 82.35%) were identified in the 36 accessions. The analyzed results of genetic diversity parameters showed that the initial collection of *A. gigantifolia* germplasm resource had large genetic diversity at a molecular level (*Na* = 1.823 5, *Ne* = 1.510 1, *He* = 0.296 5, *I* = 0.441 7). The cluster dendrogram (UPGMA) and principal coordinate analysis (PCoA) clearly showed the genetic relationship among all germplasms. All the germplasms and genetic information not only provided valuable raw materials to construct core collection of *A. gigantifolia*, but also provided the information for selecting and perserving the quality of this medicinal plant.

In conclusion, the results in this study indicated a great level of genetic variation among Guangxi wild *A. gigantifolia* germplasm. The majority of these natural accessions from similar or adjacent regions were clustered into one group with a few exceptions. Those accessions growing in similar environment generally were also clustered together. This result indicated that differentiation of *A. gigantifolia* gene pools from different regions might have resulted from reproductive isolation and divergent natural selection arising from wide geographic separation. However, there were still a few accessions showed a great difference from the majority of accessions, which might be due to the diffusion of introduced species or the introgression. We assume that human activities maybe one possible reason for the anomaly. These accessions were collected from their original po pulations and may have been cultivated in different places. It is important to collect and protect natural po pulations of *A. gigantifolia* in Guangxi, and contamination or difusion of introduced species must be avoided. In addition, in order to broaden genetic bases and improve genetic depression of the cultivated accessions, it is necessary to select wild accessions with greater genetic differences as parent accessions for cross-breeding, and these combinations might have potential for impro-

ving the quality and yield of the cultivars.

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