GENETIC DIVERSITY AND INDUCED MUTATION OF JOB'S TEARS (*Coix lachryma-jobi* L.)

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ความหลากหลายทางพันธุกรรมและการชักนำให้เกิดการกลายพันธุ์ของลูกเดือย (*Coix lachryma-jobi* L.)

นายกัง เชิน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
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GENETIC DIVERSITY AND INDUCED MUTATION OF JOB'S TEARS (*Coix lachryma-jobi* L.)

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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GANG SHEN : GENETIC DIVERSITY AND INDUCED MUTATION OF JOB'S TEARS (*Coix lachryma-jobi* L.). THESIS ADVISOR : TEERAYOOT GIRDTHAI, Ph.D., 147 PP.

MORPHERLOGICAL TRAIT/ISSR MARKER/MUTATION BREEDING/JOB'S TEARS

Genetic diversity evaluation among germplasms is an important prerequisite in Job's tears breeding program. This research aimed to study the genetic diversity and to induce mutation of Job's tears in order to apply germplasm for the breeding program. Three sets of experiments were conducted in this study.

The first experiment was carried out to study morphological diversity and to identify the relationships between traits. Ninety-four accessions collected from different provinces of China were used. The results showed a high variation among the studied materials. Relationships among traits were found which indicated that some traits could be used as an indirect selection for accession evaluation. Based on principal component (PC) analysis, 7 PCs can summarize the vast majority of the information on agronomic traits with accumulative contribution of 87.31%. Cluster analysis grouped 94 accessions into seven clusters, which revealed that genetic variation was based on types of variety, geographical distribution, and morphological characteristics.

The second experiment was conducted to evaluate genetic diversity of 94 Job's tears accessions based on 10 ISSR primers. The result found that all the primers produced 116 bands, of which 98 were polymorphic (84.48%). Guizhou population

had highest genetic diversity, whereas the lowest genetic diversity was found in Hebei population. Both value of Gst and results of AMOVA illustrated that the major proportion existed within the populations, and the minor variations existed among the populations. Genetic relationship between Guizhou and Chongqing populations was the closest, whereas the farthest occurred between Hubei and Hunan. The result of UPGMA cluster analysis among the populations was consistent with that of genetic distance. The results of both Bayesian and UPGMA cluster analysis were largely consistent despite minor differences. There was no correlation between genetic distance and geographic distance.

The third experiment was conducted to explore the effect of EMS and ^{60}Co-γ radiation on Job's tears mutagenesis. The results showed that different dose of ^{60}Co-γ radiation and concentrations of EMS had a significant impact on seed germination, seedling heights, and mutation rate. The LD50 of ^{60}Co-γ radiation irradiated for CDT and Y159 varieties were 406.305 and 284.795 Gy, respectively, and the LD50 of EMS treated for Y159 variety was 2.45% in concentration. Cluster analysis revealed that both gamma-irradiated and EMS treated samples had higher genetic variations.

Genetic patterns of Job's tears accessions obtained from this study can be helpful for breeders in parental selection. Also, ^{60}Co-γ radiation and EMS can be used in Job's tears breeding program.

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นายกัง เชิน : ความหลากหลายทางพันธุกรรมและการชักนำให้เกิดการกลายพันธุ์ของลูกเดือย (*Coix lachryma-jobi* L.) (GENETIC DIVERSITY AND INDUCED MUTATION OF JOB'S TEARS (*Coix lachryma-jobi* L.)) อาจารย์ที่ปรึกษา : อาจารย์ ดร. ธีรยุทธ เกิดไทย, 147 หน้า.

การประเมินความหลากหลายทางพันธุกรรมของเชื้อพันธุ์เป็นสิ่งสำคัญ และควรทำเป็นอันดับแรกในโปรแกรมการปรับปรุงพันธุ์ลูกเดือย งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาความหลากหลายทางพันธุกรรมและการชักนำให้เกิดการกลายพันธุ์ลูกเดือยที่สามารถประยุกต์ใช้ในโปรแกรมการปรับปรุงพันธุ์ แบ่งออกงานวิจัยเป็น 3 การทดลองดังนี้

งานทดลองแรกมีวัตถุประสงค์เพื่อศึกษาความหลากหลายทางพันธุกรรมของลักษณะทางสัณฐานวิทยา และหาความสัมพันธ์ของลักษณะต่างๆ จากลูกเดือยจำนวน 94 สายพันธุ์ที่รวมรวมมาจากมณฑลต่างๆ ของประเทศจีน ผลการทดลองพบว่าสายพันธุ์ลูกเดือยที่นำมาศึกษามีความหลากหลายทางพันธ์กรรมสูงและพบสหสัมพันธ์ระหว่างลักษณะต่างๆ ที่ศึกษาชี้ให้เห็นว่าบางลักษณะสามารถใช้ในการคัดเลือก ทางอ้อมในการประเมินสายพันธุ์ได้ จากการวิเคราะห์องค์ประกอบหลัก (principal component analysis) พบว่ามี 7 องค์ประกอบสามารถอธิบายลักษณะทางการเกษตรที่ตรวจวัดคิดเป็น 87.31 เปอร์เซ็นต์ งานทดลองนี้สามารถแบ่งลูกเดือยทั้งหมดออกเป็น 7 กลุ่มตามความแตกต่างระหว่างชนิดของพันธุ์ ภูมิประเทศและลักษณะทางสัณฐานวิทยาที่ตรวจวัด

งานทดลองที่สองมีวัตถุประสงค์เพื่อประเมินความหลากหลายทางพันธุกรรมของลูกเดือย 94 สายพันธุ์โดยใช้เครื่องหมายพันธุกรรมชนิด ISSR จำนวน 10 ไพรเมอร์ จากการทดลองพบว่าไพรเมอร์ทั้งหมดทำให้เกิดแถบดีเอนเอจำนวน 116 แถบและมีความแตกต่างของแถบดีเอนเอ 98 แถบ (84.48%) และประชากรจาก Guizhou มีความหลากหลายทางพันธุกรรมมากที่สุด ส่วนประชากรจาก Hebei มีความหลากหลายทางพันธุกรรมน้อยที่สุด จากการวิเคราะห์ค่า Gst และความแปรปรวนทางพันธุกรรม (AMOVA) แสดงให้เห็นว่าความแตกต่างทางพันธุกรรมภายในประชากรมีมากกว่าความแตกต่างทางพันธุกรรมระหว่างประชากร โดยประชากรจาก Guizhou และ Chongqing มีความคล้ายคลึงกันทางพันธุกรรมที่สุด ส่วนประชากรจาก Hubei และ Hunan มีความแตกต่างกันมากที่สุด จากการทดลองยังพบว่าการวิเคราะห์การจัดกลุ่มแบบ UPGMA มีความสอดคล้องกับผลการวิเคราะห์ระยะห่างทางพันธุกรรม นอกจากนี้การจัดกลุ่มแบบ UPGMA และ Bayesian ยังให้ผลคล้ายคลึงกัน งานทดลองนี้ไม่พบความสัมพันธ์ระหว่างระยะห่างทางพันธุกรรมและระยะห่างทางภูมิศาสตร์ของสายพันธุ์ที่ศึกษา

งานทดลองที่สามมีวัตถุประสงค์เพื่อศึกษาผลของรังสีแกรมมาและสารละลาย Ethyl methanesulfonate (EMS) ต่อการชักนำให้เกิดการกลายพันธุ์ในลูกเดือย จากการทดลองพบว่าระดับความเข้มข้นของสารละลาย EMS และรังสีแกรมมามีผลต่อความงอก ความสูงของต้นกล้า และอัตราการกลายพันธุ์ ค่าความเข้มข้นที่ทำให้เกิดการตาย 50 เปอร์เซ็นต์ (LD50) ของการฉายรังสีแกรมมาในสายพันธุ์ CDT และ Y159 มีค่าเท่ากับ 406.305 และ 284.795 Gy ตามลำดับ และค่า LD50 จากการใช้สารละลาย EMS สำหรับพันธุ์ Y159 มีความเข้มข้นเท่ากับ 2.45 เปอร์เซ็นต์ จากการวิเคราะห์การจัดกลุ่มพันธุ์ (Cluster analysis) พบว่าการใช้รังสีแกรมและสารละลาย EMS ทำให้เกิดความหลากหลายทางพันธุกรรมมากขึ้น

รูปแบบของลักษณะทางพันธุกรรมของลูกเดือยจากการทดลองนี้สามารถช่วยนักปรับปรุงพันธุ์ในการคัดเลือกสายพันธุ์พ่อแม่เพื่อใช้ในโปรแกรมการปรับปรุงพันธุ์ และยังพบว่าสารละลาย EMS และรังสีแกรมมาสามารถนำไปใช้ในโปรแกรมการปรับปรุงพันธุ์ของลูกเดือยได้

สาขาวิชาเทคโนโลยีการผลิตพืช ลายมือชื่อนักศึกษา Gang Shen

ปีการศึกษา 2560 ลายมือชื่ออาจารย์ที่ปรึกษา

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LIST OF ABBREVIATIONS

AFLP	=	Amplification Fragment Length Polymorphism
AMOVA	=	Analysis of molecular variance
cm	=	centimeter
CRD	=	Completely Randomized Design
EMS	=	Ethyl Methane Sulfonate
GNP	=	Grain Number per Plant
Gst	=	Coefficient of Gene Differentiation
He	=	Nei's Gene Diversity Index
Hs	=	Gene Diversity with Provenance
Ht	=	Total Gene Diversity
I	=	Shannon's Information Index
ISSR	=	Inter-Simple Sequence Repeats
MEGA	=	Molecular Evolutionary Genetics Analysis
ml	=	milliliter
Na	=	Observed number of Alleles
Ne	=	Effective number of Alleles
Nm	=	Estimate of Gene Flow from Gst
NPB	=	Number of Polymorphic Bands
NB	=	Number of branches
PBN	=	Primer Branch Nodes

LIST OF ABBREVIATIONS (Continued)

PC	=	Pericarp Color
PCA	=	Principal Component Analysis
PCR	=	Polymerase Chain Reaction
PH	=	Plant Height
PB	=	Panicle branch number
PPB	=	Percentage of Polymorphic Bands
RAPD	=	Random Amplified Polymorphic DNA
RAPD	=	Random Amplified Polymorphic DNA
SNN	=	Stem Node Number
SSR	=	Simple SequenceRepeats
STS	=	Sequence Tagged Sites
SW	=	100-Seeds Weight
TBC	=	Total Bract Color
TBS	=	Total Bract Shape
TBSC	=	Total Bract Surface Characteristics
TBT	=	Total Bract Texture
w/v	=	weight / volume
µl	=	microliter

CHAPTER I

INTRODUCTION

1.1 Introduction

Job's tears (*Coix lachryma-jobi* L.) which belongs to the coix genus, the Andropogononeae tribe, and the Gramineae family (Zhou et al., 2010), also known as coixseed, tear grass, hato mugi, and adlay, which is a tall grain-bearing tropical plant (Taylor, 1953). Job's tears is an annual crop planted mainly in southeast of Asian countries including China, Vietnam, Laos, Japan, and Korea (Diao et al., 2016). Job's tears seeds with sweet taste is a good ingredient for eating and treating diseases, it becomes an ideal health food and effective medicine (Gu et al., 1999; Liu, 2003). Job's tears seed can reduce fever, invigorating the spleen, diuresis, anti-cancer, hypolipidemic, hypoglycemic, antioxidant, anti-inflammatory, and anti-allergic properties (Wen, 2008; Fu et al., 2011, Xi et al., 2016). Job's tears seeds consist of good ingredient of food for eating, such as Job's tears seed tea, Job's tears seeds powder, Job's tears seeds biscuits, Job's tears seeds beverage, alcoholic and vinegar, etc. (Gu et al., 1999; Liu, 2003). The demand for Job's tears is increasing rapidly due to its medical and food, Nowadays, it has been introduced to almost all tropical and subtropical areas in the world (Lim et al., 2012).

For a long time，due to backward cultivation techniques, Job's tears was greatly affected by pests and diseases. Coupled with the long-term use of old landraces varieties, resulting the varieties appear mixed, aging and degeneration

phenomenon. In addition, because of the research work on Job's tears started late and little efforts, and the research mainly focused on its chemical components and pharmacological functions (Huang et al., 2009). Lead to the lack of new varieties with high yield and quality.

Genetic resources are the basic conditions for crop breeding, diversity analysis is an important component for efficient management and utilization of genetic resources, accurate identification of genotypes is very useful during all the steps of breeding from initial parent selection to the final utilization of cultivars in production schemes (UPOV, 1991). Moreover, diversity analysis is an essential process for clear and sound identification of the genetic relatedness of the available genetic resources. Morphological traits must be recorded for selection of parents, and always used for describing and classifying the germplasm, statistical methods including principle components or cluster analysis are useful tools for screening the accessions (Karimi et al., 2009). Morphological marker is one of the most important methods to study genetic diversity, but they are subject to environmental influences, time-consuming and must be assessed during a fixed vegetative phase of the crop life cycle (Swanepoel, 1999). Molecular markers have significant advantages over morphological markers because they are uninfluenced by growth and environmental conditions and can be applied from any growth phase (Swanepoel, 1999). Compare with other molecular markers, inter-simple sequence repeat (ISSR) is attractive method because it requires no previous DNA sequence information and is highly variable, reproducible, and cost effective. ISSR has been widely used to study the genetic diversity of various plants (Rodrigues et al., 2013).

There are lack a of international journal reported about breeding programs of Job's tears, and the main strategies used in breeding Job's tears is pedigree selection

method. Although there are several reports on cross breeding and distant hybridization between Job's tears and its wild varieties, only a few new cultivars have been released (Li et al., 1997). Mutation breeding consists mainly of physical and chemical mutation. The use of ionizing radiation, such as X-rays, gamma rays and neutrons and chemical mutagens for inducing variation, are well established. Gamma ray irradiation may cause changes in physiology and biochemistry of mutants because it contains a large amount of kinetic energy causing structural changes in the chromosomes of plants. Gene mutations can lead to the emergence of different phenotypes from mother plants and can be inherited (Encheva，2009；Morad et al.,2011), it is most commonly used and most favorable for the induction of mutants (Ahloowalia et al.,2004). Ethyl methane sulfonate (EMS) is the most widely used and most obvious application of a chemical mutagen in the crop mutation breeding, it has high mutagenic efficiency, high frequency and wide range compared with other mutagens (Lebkowski et al.,1986). Hence, gamma irradiation and Ethyl methane sulfonate (EMS) breeding technique will be used in breeding of Job's tears.

Due to the limitation of material resources, the knowledge on genetic diversity and breeding of Job's tears is still limited and the lack of new excellent varieties are still existed. Through analysis of the genetic diversity of accessions, clarifying the genetic relationships among the accessions and selection of excellent accessions as parents for breeding would be the most important ways in breeding of Job's tears. The results can provide theoretical basis and practical experience for future study in Job's tears breeding, and promote the development of the Job's tears industry to meet the needs of people's lives.

1.2 Research Objectives

1. To analyze the genetic diversity of 94 Job's tears accessions by morphological traits and ISSY marker.

2. To investigate the mutagenesis and polymorphism of Job's tears by using ISSR marker, and determine suitable mutagenic dose for mutation breeding.

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CHAPTER II

REVIEW OF THE LITERATURE

2.1 General information of Job's tears

Job's tears (Coix *lachryma-jobi* L.) belongs to the Coix genus, the Andropogononeae tribe, and the Gramineae family (Zhou et al., 2010), also known as coix seed, tear grass, hato mugi, and adlay, is a tall grain-bearing tropical plant (Taylor, 1953) and an annual crop planted mainly in Asian countries including China, Vietnam, Laos, Japan and Korea (Diao et al., 2017). In taxonomy, Job's tears has four varieties that are accepted by the World Checklist of Selected Plant Families (The species was named by Carl Linnaeus in 1753). The first variety is *Coix lacryma-jobi* var. *Lacryma-jobi*. The second variety is *Coix lacryma- jobi* var. *ma-yuen* (Ro-m. Caill.) Stapf. The third variety is *Coix lacryma-jobi* var. *puellarum* (Balansa) A.Camus. The fourth variety is *Coix lacryma-jobi* var. *stenocarpa* Oliv, which distributed to Eastern Himalayas to Indochina (World Checklist of Selected Plant Families, 2015).

Job's tears have different levels of ploidy on the basic chromosome number x=5, such as diploids (2n=10), tetraploids (2n=20), hexaploids (2n=30), and octoploids (2n=40) (Clayton, 1981). A few cases of aneuploid have also been reported. The species *C. lacryrna-jobi* L., commonly called Job's tears (2n=20), is an exclusively cultivated species and is widely used as food and medicine in eastern and southern Asian countries (Woo et al., 2007).

There are several medicinal functions and biological active components have been investigated recently in different parts of Job's tears, including seed, root, hull, bran, testa and endosperm. Job's tears seeds with sweet taste can reduce fever, invigorate the spleen, diuresis, diminish inflammation, relieve pain, alleviate a swelling, moisturize the skin, improve looks, remove fatigue, guard against hypertension and stimulate digestion (Fu et al., 2011). Especially coixenolide ($C_{38}H_{70}O_4$) extracted from Job's tears seeds have the function to stunt the growth of cancer cells (Wen, 2008). Researchers investigated the antiulcer activity of Job's tears seed and demonstrated that caffeic acid was one of the compounds of gastro protective agent (Chung et al., 2011). They also demonstrated the ethyl acetate fraction of Job's tears bran ethanolic extract retard carcinogenesis through an anti-inflammatory pathway, and potential active component was ferulic acid (Chung et al., 2010). Job's tears extracts can increase the activity of cytotoxic T lymphocytes and natural killer cells in experimental animals (Hidaka et al., 1992), and it have been studied to be antiproliferative and chemopreventive on lung or colon cancer in vivo and in vitro (Hung et al., 2003; Chang et al., 2003; Shih et al., 2004). Job's tears also has a modulating ability to shift the balance from Th2 to Th1 dominance in the T-cell-mediated immune response and may be beneficial for the treatment of allergic disorders (Kuo et al., 2001; Hsu et al., 2003). Job's tears seeds diet therapy plays a role in improving the nutritional status of peritoneal dialysis patients by relieving digestive tract symptoms, increasing urinary volume, and meliorating micro-inflammatory state (Wu et al., 2014). The study results indicated that dietary supplementation with Job's tears is likely to reduce the risk of coronary heart disease related to hypercholesterolemia and oxidative stress (Wang et al., 2012).

In addition to, Job's tears is efficient in removal of N and P from polluted water, it is generally preferred to use native plants in CW (Constructed Wetlands） systems as they are adapted to the local climate and usually have an inherent high tolerance to fungi and insect pests (Xu et al., 2007). Job's tears has a preference for NO_3^- which makes it suitable for wastewater treatment in constructed wetland system where a substantial fraction of the N is on NO_3^- form such as in vertical flow constructed wetlands (Arunothai et al. 2013).

2.2 The production of Job's tears in world

Because of its nutritional and health values, Job's tears is increasingly utilized as food and drink now, it becomes an ideal health food. Various foods in which coix seed serves as an ingredient has been developed, such as Job's tears seeds tea, Job's tears seeds powder, biscuits, dilated food, beverage, alcoholic and vinegar (Liu et al., 2003; Gu et al., 1999; Zhang et al., 1995). In Korea, a thick drink called *yulmu cha* (literally Job's tears tea) is made from powdered of Job's tears seeds. A similar drink, called *Yi Ren Jiang*, also appears in Chinese cuisine; In Thailand, it is often consumed in teas and other drinks, such as soy milk; In Korea and China, distilled liquors are also made from the grain. One such example is the South Korean liquor called *okroju*, which is made from rice and Job's tears; In Japan, an aged vinegar is made from the Job's tears grains (Arora, 1997).

2.3 Genetic diversity of Job's tears

The only systematic germplasm collection of Job's tears was conducted in China. In 1981, 250 accessions of landrace and wild types were collected from 17

provinces (Diao, 2016). Based on growth period and other characteristic, the Chinese Job's tears germplasm was classified into three ecotypes: the southern China late-maturation type, the middle and low Yangtze River region medium-maturation type, and the northern China early maturation type (Huang et al., 1995). Comparing the genetic diversity of 77 accessions of Job's tears from China, Japan and South Korea, the results shows that there is a clear difference among the Job's tears germplasm in plant height, leaf area, effective tiller number, stem diameter and other morphological and growth characteristics; The results also indicates that Job's tears both from Japan and South Korea can't grow very well, so it is not suitable to grow in southern of China, and have little significance to applicate directly in the production (Liang et al., 2006).

The genomic DNA polymorphisms of 66 accessions of Job's tears were analyzed with RAPD (random amplified polymorphic DNA) techniques, the result showed that 3 groups of Job's tears were determined based on cluster analysis, similar results were found from mix-DNA and the survey of agronomic characters of Job's tears suggesting that the mix-DNA for phylogenetic analysis might be an effective way for studying genetic relationship of Job's tears (Su et al., 2008).

The genetic diversity of 22 Job's tears accessions were studied by simple sequence repeats (SSR) markers. eleven SSR primers giving stable amplified band pattern detected 105 alleles among the lines tested. The average number of alleles per SSR locus was 9.55 with a range from 4 to 20. The value of polymorphism information content (*PIC*) for each SSR locus varied from 0.3048 to 0.9238 with average of 0.8255, and the 22 accessions were divided into 4 groups by using SSR fingerprinting, the clustered results were similar to that based on geographical resource

and germplasmic genealogy, the result also indicated that SSR marker could be an accurate and reliable method to study diversity of Job's tears, and could assist genetic improvement of Job's tears breeding (Guo et al., 2013). The genetic diversity and relationships among 79 Job's tears (*Coix lacryma-jobi* L.) accessions which collected from China and Korea by using 17 microsatellite markers, the results shows that total of 57 alleles were detected with an average of 3.4 alleles per locus, a high frequency of rare alleles (36.3 %) was observed within the collection, the values for observed (HO), expected heterozygosity (HE) and Shannon's information index (I) within the analysis ranged from 0.00 (GBssrJT183) to 0.81 (GBssrJT130), from 0.01 (GBssrJT170) to 0.65 (GBssrJT130) and from 0.034 (GBssrJt170) to 1.13 (GBssrJT130), respectively; Based on the UPGMA algorithm, the majority of the Chinese accessions grouped in one cluster, whereas all the Korean accessions grouped together in a separate cluster (Ma et al., 2010).

Studied on DNA diversity of 42 accessions, which were studied by 36 pairs Sequence Tagged Sites (STS)primer according to the sequences of shattering related genes for polymerase chain reaction (PCR). Among them, there were five pairs of primers with good stable polymorphism, which were used for establishing the STS fingerprint map and a phylogeny tree，the evolutionary relationship was also discussed (Jiang et al., 2013).

2.4 Breeding of Job's tears

2.4.1 The development of hybridization breeding

Two different Job's tears (*coix lacryma-jobi* L. and *Coix lacryma-jobi* L. *friumentacea* Makino) were used to develop inbred and hybrid experiment, the

result shows that in F_1 generations, height, leaf area, growth, photosynthetic characteristics exhibited heterosis, but appeared pollen sterility, and the pollen abortion rate is up to 79%, the seed setting rate is also low, only about 28%, and hybrids instability, the traits separate very serious and not stable until F_7 generations (Qiao et al., 1993). The same experiment was done to study F_1 and their parents, the result showed that some characteristics like resistance and quality of *Coix lacryma-jobi* L. were expressed in F_1, the morphology, growth potential and yield have comparative advantage in F_1, but there are some degree of infertility in F_1, and the seed setting rate from 30% to 50% (Du et al., 1998). By using same parents for hybridization, after eight years of selection, two relatively stable new line of hybrids were obtained (cv.85-15 and cv. 95-18), the hundred-grain weight is 14.4g and 15.9g, the yield is 7,545.0 kg per hectare and 8,545.0 kg per hectare, respectively, and the new hybrid line has a strong anti-smut, resistant to leaf blight and tolerance to cold (Li et al., 1997). The crossing experiment using *Coix. agrestis* Lour. With white stigmas and C. *lacryma-jobi* L. with red stigma shows that the stigma color of F_1 generation is purple, F_2 generation characters appear separation, and the proportion of white and purple stigma plants is 9:7. And result shows that the ancestors of Job's tears stigma may be purple (Du et al. 1998).

Hybrid experiment between maize and genus Job's tears, using maize as male, Job's tears as female, F_1 generations can be obtained, but failed to get the hybrid offspring from reciprocal cross (Haradc et al., 1954). Using Job's tears pollen as materials by fluorescence microscopy, put the Job's tears pollen on the stigma of maze, then observe their germination and growth, the results showed that Job's tears pollen grains can germinate on the stigma of maze, the pollen tube can also insert into the

stigma of maze**.** However, due to the presence of reproductive isolation between maize and Job**'**s tears, it is extremely difficult to hybridize between maize and Job**'**s tears**.** (Duan et al., 2008).

2.4.2 The mutation breeding of Job's tears

Availability of genetic variability is the prerequisite for any breeding program. Besides conventional methods, induced mutation has been extensively used for creating new genetic variation in crop plants. The use of ionizing radiation, such as X-rays, gamma rays and neutrons and chemical mutagens for inducing variation, is well established. Chemical and ionizing radiation mutagenesis have been routinely used to generate genetic variability for breeding research and genetic studies. More than 2200 crop varieties were released by the end of the last century using irradiation mutagenesis (Maluszynski et al., 2003). In many mutagenic studies, gamma ray and X-rays have been used to induce mutations. The key factor in the irradiation of plant material is the dose, which is the amount of radiation energy absorbed by the material**.** The unit of measurement of radiation dose is Gray (Gy). One Gy is equal to the absorption of 1J of energy per kilogram of product irradiated**.** Radiation doses are divided into three broad categories; high (> 10 kGy), medium (1 to 10 kGy), and low (<1 kGy). The high doses are used for the sterilization of food products, and low doses to induce mutations in seed material, where doses range from 60 to 700 Gy for many seed propagated crops, such as rice, wheat, maize, beans and rape seed.

Several positive mutations have been created in agricultural crops by the use of gamma irradiations. Crops with improved characteristics have successfully been developed by mutagenic inductions (Rehman et al., 1987; Javed et al., 2000). Khatri et al**.**, (2005) collected three high grain yielding and early maturing mutants by treating

seeds of Brassica juncea L. cv. S-9 with gamma rays (750-1000KGy). Shah et al. (2001) developed a new oil seed *Brassica napus* L cv. ABASIN-95 by induced mutation. (Majeed and Muhammad, 2010) investigated effects of gamma rays on days to germination, days to completion of germination, germination percentage, survival percentage, shoot length/plant, root length/plant, number of branches/plant, number of leaves/plant, fresh weight of shoot/plant and dry weight of shoot/plant of *Lepidium sativum* L. Dry seeds of *Lepidum sativum* L were irradiated with 20, 30, 40, 50, 60, 70 and 80Gy) by ^{60}Co-Gamma irradiation**.** The study revealed that gamma irradiation significantly affected all the mentioned parameters except germination percentage. Days to initiation of germination and days to completion of germination were significantly delayed at higher doses of gamma rays. However, other growth parameters showed declining tendency with increasing doses of gamma irradiation. Germination percentage was not significantly affected by higher doses of gamma rays.

Using ^{60}Co-γ ray irradiation treatment the F_1 of Job's tears, the dwarf, precocious and other mutants can be obtained (Du et al., 2002). Using different doses of ^{60}Co-γ ray irradiation (150, 250, 350, 450, 550, 650, and 750 GY) treatment three local varieties of Job's tears which from medicinal materials production based in ZheJiang province, two high-quality, high yield and dwarf Job's tears new strain have been selected, the yield of new strain were higher 10.9% and 14.1% than local varieties which have best yield in local, the thousands of grain increase 13.9% and 15.8%, the content of glycerol trioleate increase 6.5% and 9.6% (Shen, 2007).

Experiments with chemical mutagens were at variance with those of physical mutagens**.** As compared with physical mutagens, chemicals may give rise to relatively more gene mutations rather than to chromosomal changes**.** The assessment of LD_{50} for

chemicals is determined by varying the concentration and duration of treatment, the solvent used, or the pH of the solution (Novak, 1991). Among the chemical mutagens, alkylating agents, especially EMS was demonstrated to be widely used, and have been proved to be the most effective with little side effects**.** At present, EMS is the most widely used and most obvious application effect of a chemical mutagen in the crop mutation breeding, it has high mutagenic efficiency, high frequency and wide range compared with other mutagens (Lebkowski et al., 1986). It has been applied and obtained valuable mutation in rice (Gu, 2005), wheat (Du, 1900), soybean (Wang et al., 1993), corn (Neuffer, 1978) and other crops. EMS was used for inducing mutations in banana by treating shoot tips and then regenerating adventitious buds. The best responses were achieved with 24.69 mM and 3 h of incubation**.** EMS was also used on in vitro cultures of apple (*Malus domestica Borkh.*). Variants for vigor, floral precocity, and fruit set were then selected in the field (Webster et al., 1985).

Ethyl methane sulfonate (EMS) mutation for Job's tears, by using different concentrations of EMS solution (0.3%, 0.6%, 0.9%) treated coix seed for 6h. The results indicated that EMS could induce chromosomal aberration of root tip cell and different damage effect of seeding ratio, seedling height, root length, root number and peroxidase activity (Yuan et al, 1996).

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CHAPTER III

PRINCIPLE COMPONENT AND MORPHOLOGICAL DIVERSITY ANALYSIS OF JOB'S TEARS (*Coix lacryma-jobi* L.)

3.1 Abstract

The diversity analysis of Job's tears, an ideal healthy food crop, is prerequisite in breeding program and gerplasm utilization. The aim of this study was to characterize the quantitative and qualitative phenotypic traits of 94 Job's tears accessions (40 cultivated and 54 wild accessions) collected from different geographical areas of China. Principle component and genetic diversity analysis on 12 morphological characters were conducted. The results showed high variation among the studied materials. Relationship among traits were found indicated that these traits could be used as indirect selection for accessions evaluation. Based on principal component analysis, the first seven PCs in the experiment can summarize the vast majority of the information of agronomic traits of the Job's tears accessions, the accumulative contribution rate account for 87.31 % of the total variation. Cluster analysis grouped the 94 accessions into seven clusters, Cluster I contained 21 accessions, the accessions in this group had different plant characteristics which are good for breeding material. Cluster II contained only Y 77 with highest in plant height, the most stem node number and panicles per plant, 100-seed weight, and this accession can be used as high-yielding variety and parental material to improved

superior dwarfing variety. Cluster III can be used as excellent resources to cultivate dwarfing, anti-lodging and high yield variety. Cluster IV with most number of branches and panicle branch number can be used as excellent parental material to improve yield. Cluster VII can be used as excellent parent material for cultivated dwarfing and anti-lodging new varieties. The results also revealed that genetic variation was based on types of variety, geographical distribution, and morphological characteristic.

3.2 Introduction

Job's tears (*Coix lachryma-jobi* L.) is an annual and important economic crop planted mainly in Asian countries including China, Vietnam, Laos, Japan and Korea (Diao et al., 2017). It belongs to the Coix genus, the Andropogoneae tribe within the family Poaceae with a tall grain-bearing tropical plant (Taylor, 1953, Zhou et. al., 2010). Job's tears also known as coix seed, tear grass, hato mugi, and adlay. The demand in Job's tears is increasing rapidly due to its medicinal functions as an ideal healthy food. As a result, it has been introduced and grown almost all tropical and subtropical areas in the world (Lim et al., 2012).

Due to an impropriated cultivation techniques, coupled with long term use of low-yielding landraces varieties causes low seed quality and production. In addition, a few research works have been conducted on Job's tears. Most research mainly focused on its chemical components and pharmacological functions lead to the lack of new varieties with high yield and quality (Huang et al., 2009). Genetic resource is the most important part for plant breeding program and diversity analysis is prerequisite for more efficient management and utilisation of genetic resources. Accurate

identification of the genotype is very improtance during all the steps of breeding from initial parent selection to the final utilisation of cultivars in production schemes (UPOV, 1991). Moreover, diversity analysis is an essential process for clear identification of the genetic relatedness of the available genetic resources. Modern objectives in plant breeding might be achieved by the evaluation of traits within genetic resources. Although, molecular markers methods for genotype description have been proved useful, but these methods are expensive and needed marker-linked traits information. Morphological characters must be recorded for selection of parents and their progenies, and always be used for describing and classifying the germplasm. Principle components and cluster analysis are also useful tools for screening the accessions (Karimi et al., 2009). For better conserve and utilize genetic resources, appropriate variables of morphological should be carefully chosen (Giraldo et al., 2010). There are some studies have reported the morphological analyzed the accessions of Job's tears. Ten major quantitative traits of 12 Job's tears germplasms from different regions were analysed through path and principal component analysis (Li et al., 2010). The main components and cluster analysis of 25 Job's tears accessions were also conducted in 2013 (Wang et al., 2013). So far, studies on genetic diversity of Job's tears have been made on accessions mainly from the Guangxi, Guizhou, and Yunnan Provinces in southern China (Ma et al., 2010; Li et al., 2001; Wang et al., 2015).

Therefore, the objectives were to (1) analyze the genetic diversity of 94 Job's tears accessions from different regions in China, and (2) clarify the genetic relationships among the accessions. This will provide valuable information for

selection excellent accessions as parents for breeding and utilization, and conservation of Job’s tears genetic resource.

3.3 Materials and Methods

3.3.1 Plant materials

Ninety-four Job's tears accessions evaluated in this study were shown in Table 3.1. Forty cultivated and fifty-four wild accessions were collected from different geographical regions of seven province of China show in figure 1 and table 3.2. All Job's tears accessions were collected and multiply during 2013-2014.

Figure 1 The germplasm of Job's tears distributed in seven province of china.

Table 3.1 The source region and accession numbers of 94 Job's tears.

Source Region	**Guizhou**	**Guangxi**	**Sichaun**	**Chongqing**	**Hunan**	**Yunnan**	**Hubei**	**Qianxinan Institute**
Accession Numbers	36	19	7	7	7	5	4	9

3.3.2 Experiment

Thirty seeds per accession of Job's tears were immersed in 60°C hot water for 30 minutes, and then planted in the seedling cups. Seedlings with 3-4 leaves were transplanted to the field at the Germplasm Resources Garden of Guizhou Academy of Agricultural Sciences (altitude at 1,074.3 meters above sea level, with latitude and longitude of 106.71 and 26.57, respectively), in Guizhou province of the China, during April 2015 and April 2016. Completely Randomized Design (CRD) with 3 replications was employed. Each replication consisted of 10 plants with 1 plant per hill. The row and plant spacing of each plant were 60 cm and 40 cm, respectively.

3.3.3 Crop management

Phosphate Fertilizer 75,000 kg/hectare, Potash Fertilizer 15,000kg/ hectare, Urea 15,000kg/ hectare as Base Fertilizer. 45,000 kg/hectare of compound fertilizer was applied after 30 cm in seedling height. Then 2% superphosphate solution was used for extra-root dressing at flowering stage.

Weeding for two times. The first weeding was carried out at a plant height of 20 cm; The second weeding was carried out with the fertilization and soil cultivation at a plant height of 30 cm.

Seedling stage, panicle stage, flowering stage and grain filling stage should ensure that there is enough water. In case of drought, it is necessary to water in

the early evening to keep the soil moist. After the rain and furrow irrigation, water should be removed. At the same time prevent smut, leaf blight, corn borer and other pests.

3.3.4 Data collection

Twelve different quantitative and qualitative traits were evaluated at harvest time. Five plants (normal growth, uniform performance with diseases and insect pests free) of each accession were randomly selected for scoring. Seven quantitative characters including the stem node number (SNN, from the first node at the base to the last node at the top), number of branches (NB), primer branch nodes (PBN, ordinal number of node with first panicle branch appears), panicle branch number (PB), grain number per plant (GNP) were evaluated. The plant height (PH) was measured from ground to tip of the main spike at physiological maturity. Hundred-seed weight (SW) was measured with a scale sensitive to 0.1g). Five qualitative characters were scored according to Li Chunhua 2015 (Li et al., 2015) (total bract surface characteristics(TBSC): smooth=1,longitudinal convex stripes=2, total bract texture (TBT): enamel=1,crustaceous =2, total bract shape (TBS): circular =1, oval =2, length circle =3, total bract color(TBC):white =1, greyish white =2, gray blue without dark stripes =3, gray blue with dark stripes =4, yellow white =5, tawny without dark stripes =6, tawny with dark stripes=7, brown =8, dark brown=9, pericarp color(PC): light yellow =1, yellow =2, brown =3).

3.3.5 Data analysis

For the 7 quantitative traits, descriptive statistics were computed for each accession by using the Statistical Package for Social Science (SPSS 20.0 Inc., USA). The mean, maximum, minimum, standard deviation (S.D.) and coefficient of variation (CV) were calculated. Principal component analysis was carried out by using

the original numerical data of quantitative characters and the assigned qualitative traits data. The Euclidean distance between Job's tears accessions based on morphological characteristics was calculated using between-group linkage method in SPSS20.0.

Table 3.2 The codes and collection place of Job's tears accessions used in this study.

Accession	Collection place	type	Accession	Collection place	type
Y_1	Qianjiang Chongqing	Wild	Y_{48}	Cili Hunan	Wild
Y_2	Qianjiang Chongqing	Wild	Y_{49}	Cili Hunan	Wild
Y_3	Qianjiang Chongqing	Wild	Y_{50}	Cili Hunan	Wild
Y_4	Banan Chongqing	Wild	Y_{51}	Enshi Hubei	Wild
Y_5	Nanchuan Chongqingg	Wild	Y_{52}	Shizong Hubei	Cultivated
Y_6	Nanchuan Chongqing	Cultivated	Y_{53}	Xianfeng Hubei	Wild
Y_7	Wansheng Chongqing	Wild	Y_{54}	Xianfeng Hubei	Wild
Y_8	Changshun Guizhou	Wild	Y_{55}	Longlling Guangxi	Cultivated
Y_9	Changshun Guizhou	Wild	Y_{56}	Longling Guangxi	Wild
Y_{10}	Changshun Guizhou	Wild	Y_{57}	Xilin Guangxi	Cultivated
Y_{11}	Zunyi Guizhou	Wild	Y_{58}	Baise Guangxi	Cultivated
Y_{12}	Huaxi Guizhou	Wild	Y_{59}	Tianlin Guangxi	Cultivated
Y_{13}	Huaxi Guizhou	Wild	Y_{60}	Qianxinan Institute1	Cultivated
Y_{14}	Huaxi Guizhou	Wild	Y_{61}	Qianxinan Institute2	Cultivated
Y_{15}	Huaxi Guizhou	Wild	Y_{62}	Qianxinan Institute3	Cultivated
Y_{16}	Suiyang Guizhou	Wild	Y_{63}	Qianxinan Institute4	Cultivated
Y_{17}	Ziyun Guizhou	Cultivated	Y_{64}	Qianxinan Institute5	Cultivated
Y_{18}	Yuping Guizhou	Wild	Y_{65}	Qianxinan Institute6	Cultivated
Y_{19}	Meitan Guizhou	Wild	Y_{66}	Qianxinan Institute7	Cultivated
Y_{20}	Wangmo Guizhou	Wild	Y_{67}	Qianxinan Institute8	Cultivated
Y_{21}	Wangmo Guizhou	Wild	Y_{68}	Qianxinan Institute9	Cultivated
Y_{22}	Wangmo Guizhou	Wild	Y_{69}	Zizhong Sichuan	Wild
Y_{23}	Wangmo Guizhou	Wild	Y_{70}	Zizhong Sichuan	Wild

Table 3.2 The codes and collection place of Job's tears accessions used in this study (Continued).

Accession	Collection place	type	Accession	Collection place	type
Y_{24}	Wangmo Guizhou	Wild	Y_{71}	Jianyang Sichuan	Wild
Y_{25}	Qianlong Guizhou	Cultivated	Y_{72}	Jianyang Sichuan	Wild
Y_{26}	Puding Guizhou	Cultivated	Y_{73}	Jianyang Sichuan	Wild
Y_{27}	Puding Guizhou	Wild	Y_{74}	Chengdu Sichuan	Wild
Y_{28}	Puding Guizhou	Wild	Y_{75}	Tianjin Sichuan	Wild
Y_{29}	Sinan Guizhou	Cultivated	Y_{76}	Maguan Yunnan	Cultivated
Y_{30}	Yinjiang Guizhou	Cultivated	Y_{77}	Maguan Yunnan	Cultivated
Y_{31}	Yinjiang Guizhou	Wild	Y_{78}	Qiubei Yunnan	Cultivated
Y_{32}	Wuchuan Guizhou	Wild	Y_{79}	Xichou Yunnan	Wild
Y_{33}	Wuchuan Guizhou	Wild	Y_{80}	Luoping Yunnan	Cultivated
Y_{34}	Wuchuan Guizhou	Wild	Y_{81}	Luoping Yunnan	Cultivated
Y_{35}	Ceheng Guizhou	Wild	Y_{82}	Luoping Yunnan	Cultivated
Y_{36}	Ceheng Guizhou	Cultivated	Y_{83}	Luoping Yunnan	Cultivated
Y_{37}	Ceheng Guizhou	Cultivated	Y_{84}	Quejing Yunnan	Wild
Y_{38}	Xingren Guizhou	Cultivated	Y_{85}	Wenshan Yunnan	Wild
Y_{39}	Xingren Guizhou	Cultivated	Y_{86}	Wenshan Yunnan	Cultivated
Y_{40}	Xingrenxiashan Guizhou	Cultivated	Y_{87}	Shizong Yunnan	Wild
Y_{41}	Xingrenxiashan Guizhou	Cultivated	Y_{88}	Shizong Yunnan	Cultivated
Y_{42}	Xingyi Guizhou	Cultivated	Y_{89}	Fuyuan Yunnan	Cultivated
Y_{43}	Guanling Guizhou	Cultivated	Y_{90}	Fuyuang Yunnan	Cultivated
Y_{44}	Yongshun Hunan	Wild	Y_{91}	Fuyuan Yunnan	Cultivated
Y_{45}	Sanzhi Hunan	Wild	Y_{92}	Kunming Yunnan	Wild
Y_{46}	Sanzhi Hunan	Wild	Y_{93}	Dali Yunnan	Wild
Y_{47}	Sanzhi Hunan	Wild	Y_{94}	Lijiang Yunnan	Wild

3.4 Materials and Methods

3.4.1 Comparative analysis of 7 quantitative traits in different years

Comparing the 7 quantitative traits of 94 Job's tears accessions between 2015 and 2016, the results were shown in Table 3.3. The results showed that the seven quantitative traits were extremely significant effected by different planting years, different accessions and the year combine accessions. But the main influence comes from different accessions (most traints more than 95%) and the CV values were very small , it illustreated that environment can significant impact the quantitative traits of 94 Job's tears , but the main influence comes from the genetype.

3.4.2 Comparative analysis of 7 quantitative traits between cultivated and wild accessions

There were significant genetic variations in all quantitative traits of cultivated and wild accessions (Table 3.4). For cultivated species, the average value of plant height was 198. 6 cm ranging from 59.6 cm to 263.4 cm, the standard deviation (SD) was 32.4 cm, and the coefficient of variation (CV) was 16.3%. For the wild species, the mean value of plant height was 200.9 cm ranging from 148.7 cm to 263.3cm, the SD was 25.4 cm, and the CV was 12.6%. Thus, the variation of plant height of cultivated species was greater than that of wild species. Although the maximum value of cultivated species was similar to that of wild plants, Y35 (263.4) and Y41 (263.3), the minimum was quite different, which was 56.87 and 150, respectively. The mean variations of wild species were higher than cultivated species in number of branches, panicle branch number and 100-seed weight, while the stem node number and primer branch nodes have not much significant difference. For grain number per plant, the mean of wild species larger than cultivated species were 165.7 and 135.2, respectivly.

Table 3.3 Comparison of the Sum of Square (SS) of 7 quantitative traits between 2015 and 2016 years.

Source	df	PH	SND	NB	PBN	PB	GNP	SW
Year	1	316**	17.48**	6.5**	0.7**	1480**	1610**	26.4**
Accession	93	466768**	1015.9**	5939.0**	981.1**	334899**	2327222**	4417.5**
Year*Accession	93	7712**	101.6**	100.9**	27.5**	2800**	26673**	157.3**
Error	376	15524	39.7	164.1	31.4	2415	40377	63.7
Total	563	490320	1174.7	6210.4	1040.8	341593	2395881	4664.9
CV		3.2	3.2	6.8	6.1	4.4	7.0	3.9

PH: plant height, SNN: stem node number, NB: number of branches, PBN: primer branch nodes, PB: panicle branch number, GNP: grain number per plant, SW: 100-seed weight.

Table 3.4 The analysis of 7 quantitative morphological traits.

Item	Plant height (cm)	Stem node number	Number of branches	Primer branch nodes	panicle branch number	Grain number per plant	100-seed weight (g)
Min	59.6	7.4	5.0	1.8	19.9	45.0	6.8
	148.7	6.9	5.5	1.7	25.6	59.2	6.7
Max	263.4	14.4	15.7	8.7	100.4	316.7	19.7
	263.3	13.4	25.6	8.5	120.4	302.8	25.3
Mean	198.6	10.3	8.5	4.7	51.4	135.2	10.1
	200.9	10.0	11.1	4.5	64.4	165.7	11.0
SD	32.4	1.4	2.9	1.4	21.1	65.7	2.5
	25.4	1.5	4.0	1.4	27.5	63.3	3.3
CV %	16.3	13.2	33.7	30.3	41.0	48.7	25.2
	12.6	14.9	36.4	30.1	42.6	38.2	29.9

Uplink: Cultivated species，Downlink: Wild species.

3.4.3 Qualitative traits analysis

Qualitative traits of Job's tears germplasm resources were shown in Table 3.5 and Attached table 1. Eighty accessions have total bract surface characteristics in smooth accounting for 85.1% of the total, and 14 accessions have longitudinal convex stripes accounting for 14.9%. There were 40 accessions have crustaceous in the total bract texture accounting for 42.6% of the total, whereas 51 accessions have enamel, accounting for 57.4%. Among total bract shape, 5 accessions have circular shape accounting for 5.3%, 54 accessions have oval shape accounting for

57.4% and 35 accessions have length circle shape accounting for 37.3%. For total bract color, white, greyish white, gray blue without dark stripes, gray blue with dark stripes, yellow white, tawny without dark stripes, tawny with dark stripes, brown and dark brown accounting for 28.6, 6.4, 3.2, 3.2, 1.1, 4.3, 6.4, 20.2 and 26.6%, respectively. There were 48 accessions have light yellowing pericarp color accounting for 51.1%, the yellow of pericarp color had 12 accessions, accounting for 12.8%, and the brown of pericarp color had 34 accessions accounting for 36.1%.

3.4.4 Correlation coefficient analysis

The Pearson correlation coefficient revealed significant correlations among some variables measured in Job's teas accessions (Table 3.6). Number of branches, panicle branch number, plant height and stem node number were positively correlated with grain number per plant, wherea primer branch nodes and 100-seed weight were negatively correlated with grain number per plant. Plant height and 100-seed weight were positively correlated with number of branches, wherea primer branch nodes and stem node number were negatively correlated with number of branches. The most positive correlation occurred between primer branch nodes stem node number, wherea the negative correlation occurred between grain number per plant and 100-seed weight.

Table 3.5 Qualitative morphological traits of 94 Job's teas accessions.

Code	TBSC	TBT	TBS	TBC	PC	Code	TBSC	TBT	TBS	TBC	PC
Y1	Smooth	Enamel	Oval	Tawny without dark stripe	light yellow	Y25	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow
Y2	Smooth	Enamel	Oval	Brown	brown	Y26	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow
Y3	Smooth	Enamel	Oval	Brown	brown	Y27	Smooth	Enamel	Oval	brown	brown
Y4	Smooth	Enamel	Oval	Greyish white	brown	Y28	Smooth	Enamel	Oval	brown	yellow
Y5	Smooth	Enamel	Oval	Tawny with dark stripes	light yellow	Y29	Longitudinal convex stripes	Crustaceous	Length circle	brown	light yellow
Y6	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow	Y30	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow
Y7	Smooth	Enamel	Oval	dark brown	light yellow	Y31	Smooth	Enamel	Oval	tawny with dark stripes	light yellow
Y8	Smooth	Enamel	Oval	gray blue with dark stripes	light yellow	Y32	Smooth	Enamel	Oval	tawny with dark stripes	light yellow
Y9	Smooth	Enamel	Oval	brown	yellow	Y33	Smooth	Enamel	Oval	tawny without dark stripes	brown
Y10	Smooth	Enamel	circular	yellow white	brown	Y34	Smooth	Enamel	Oval	dark brown	brown
Y11	Smooth	Enamel	Oval	brown	brown	Y35	Smooth	Enamel	Oval	greyish white	brown
Y12	Smooth	Enamel	Oval	tawny with dark stripes	light yellow	Y36	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow
Y13	Smooth	Enamel	Length circle	tawny with dark stripes	light yellow	Y37	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow
Y14	Smooth	Enamel	Oval	dark brown	brown	Y38	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow
Y15	Smooth	Enamel	Oval	dark brown	brown	Y39	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow

TBSC: Total bract surface characteristics，TBT: Total bract texture，TBS; Total bract shape，TBC: Total bract color，FT: Fruit type，PC: Pericarp color.

Table 3.5 Qualitative morphological traits of 94 Job's teas accessions (Continued).

Code	TBSC	TBT	TBS	TBC	PC	Code	TBSC	TBT	TBS	TBC	PC
Y16	Smooth	Enamel	Oval	brown	yellow	Y40	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow
Y17	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow	Y41	Longitudinal convex stripes	Crustaceous	Length circle	dark brown	brown
Y18	Smooth	Enamel	Oval	brown	yellow	Y42	Longitudinal convex stripes	Crustaceous	Length circle	White	brown
Y19	Smooth	Enamel	Oval	dark brown	brown	Y43	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow
Y20	Smooth	Enamel	Oval	gray blue with dark stripes	light yellow	Y44	Smooth	Enamel	Oval	brown	yellow
Y21	Smooth	Enamel	Oval	tawny with dark stripes	light yellow	Y45	Smooth	Enamel	Oval	brown	yellow
Y22	Smooth	Enamel	Oval	greyish white	yellow	Y46	Smooth	Enamel	Oval	tawny without dark stripes	light yellow
Y23	Smooth	Enamel	Oval	greyish white	yellow	Y47	Smooth	Enamel	Oval	dark brown	yellow
Y24	Smooth	Enamel	Oval	dark brown	brown	Y48	Smooth	Enamel	Oval	tawny without dark stripes	yellow
Y49	Smooth	Enamel	Oval	tawny without dark stripes	light yellow	Y72	Smooth	Enamel	Oval	dark brown	brown
Y50	Smooth	Enamel	Oval	brown	yellow	Y73	Smooth	Enamel	Oval	brown	light yellow
Y51	Smooth	Enamel	Oval	brown	brown	Y74	Smooth	Enamel	Oval	dark brown	brown
Y52	Longitudinal convex stripes	Crustaceous	Length circle	gray blue with dark stripes	light yellow	Y75	Smooth	Enamel	Oval	dark brown	brown
Y53	Smooth	Enamel	Oval	Greyish white	brown	Y76	Longitudinal convex stripes	Crustaceous	circular	dark brown	light yellow
Y54	Smooth	Enamel	Oval	dark brown	light yellow	Y77	Longitudinal convex stripes	Crustaceous	Length circle	brown	brown

TBSC: Total bract surface characteristics，TBT: Total bract texture，TBS; Total bract shape，TBC: Total bract color，FT: Fruit type，PC: Pericarp color.

Table 3.5 Qualitative morphological traits of 94 Job's teas accessions (Continued).

Code	TBSC	TBT	TBS	TBC	PC	Code	TBSC	TBT	TBS	TBC	PC
Y55	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow	Y78	Longitudinal convex stripes	Crustaceous	Length circle	brown	brown
Y56	Smooth	Enamel	Oval	dark brown	brown	Y79	Smooth	Enamel	Oval	greyish white	brown
Y57	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow	Y80	Longitudinal convex stripes	Crustaceous	Oval	White	light yellow
Y58	Longitudinal convex stripes	Crustaceous	Length circle	dark brown	brown	Y81	Longitudinal convex stripes	Crustaceous	Oval	tawny without dark stripes	light yellow
Y59	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow	Y82	Longitudinal convex stripes	Crustaceous	Length circle	brown	brown
Y60	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow	Y83	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow
Y61	Longitudinal convex stripes	Crustaceous	Length circle	dark brown	brown	Y84	Smooth	Enamel	Oval	dark brown	brown
Y62	Longitudinal convex stripes	Crustaceous	circular	Gray blue without dark stripes	133.9	Y85	Smooth	Enamel	Oval	brown	light yellow
Y63	Longitudinal convex stripes	Crustaceous	Length circle	dark brown	light yellow	Y86	Longitudinal convex stripes	Crustaceous	Length circle	brown	light yellow
Y64	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow	Y87	Smooth	Enamel	circular	dark brown	light yellow
Y65	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow	Y88	Longitudinal convex stripes	Crustaceous	Length circle	dark brown	brown
Y66	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow	Y89	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow
Y67	Longitudinal convex stripes	Crustaceous	Oval	White	light yellow	Y90	Longitudinal convex stripes	Crustaceous	Length circle	White	yellow
Y68	Longitudinal convex stripes	Crustaceous	Oval	White	light yellow	Y91	Longitudinal convex stripes	Crustaceous	Length circle	White	light yellow
Y69	Smooth	Enamel	circular	dark brown	brown	Y92	Smooth	Enamel	Oval	White	brown
Y70	Smooth	Enamel	Oval	dark brown	brown	Y93	Smooth	Enamel	Oval	dark brown	light yellow
Y71	Smooth	Enamel	Oval	dark brown	brown	Y94	Smooth	Enamel	Oval	dark brown	brown

TBSC: Total bract surface characteristics，TBT: Total bract texture，TBS; Total bract shape，TBC: Total bract color，FT: Fruit type，PC: Pericarp color.

Table 3.6 Simple correlation matrix for the 7 quantitative traits.

	GNP	NB	PB	PBN	PH	SNN
NB	0.1611**					
PB	0.4364**	0.0664^{ns}				
PBN	-0.1096**	-0.0561^{ns}	0.0154^{ns}			
PH	0.2163**	0.09*	0.1971**	0.4539**		
SNN	0.0695^{ns}	-0.1833**	0.1556**	0.5049**	0.4591**	
SW	-0.1893**	0.1664**	-0.0187^{ns}	-0.0236^{ns}	-0.1181**	0.0277^{ns}

*P<0.05, ** P<0.01, ns P>0.05. PH: Plant height, SNN: Stem node number, NB: Number of branches, PBN: Primer branch nodes, PB: Panicle branch number, GNP: Grain number per plant, SW: 100-seed weight

3.4.5 Principal component analysis.

In order to fully reflect the various factors played a leading role in the comprehensive indicators, the principal component analysis (PCA) was carried out on 7 quantitative traits and 5 qualitative traits. The eigen values, contribution rate and accumulative contribution rate were also cumulated (Table 3.7). According to the standard of accumulative contribution rate of 85%, the first seven PCs in the experiment can be summarized the vast majority of the information of agronomic traits. The accumulative contribution rate accounted for 87.31 % of the total variation.

Total bract surface characteristics，total bract texture and total bract shape loaded highly in PC1. This indicated that the first principal component reflected the main factor of involucre characters with the eigenvalue of 3.60 and the contribution rate accounts for 30.03% of the total variation of the studied samples. In PC2, the

eigenvalue was 2.15 and the contribution rate accounts for 17.92% of the total morphological variation, among the accessions were mainly explained by plant height, stem node number and primer branch nodes. These indicated that the second principal component reflected the main factor of plant type. In PC3, the eigenvalue was 1.44 and the contribution rate accounted for 12.03% of the total variation, the grain number per plant and Panicle branch number loaded more in PC3. These indicated that the third principal component reflected the main factor of yield. In PC4, the eigenvalue was 1.05 and the contribution rate contributed 8.74% of the total morphological variation in these accessions with only 100-seed weight loading highly. This indicated that the forth principal component reflected the main factor of 100-seed weight. PC5 accounted for 7.69% of total variation with total bract shape loading highly. PC6 and PC7 accounted for 6.23% and 4.68%, mainly loaded pericarp color and total bract color, respectively. Generally, for the 12 morphological traits studied, PC1 and PC2 constituted 47.5% of the total morphological variation with most seeds related traits and vegetative traits.

3.4.6 Cluster Analysis

According to the results of clustering (Figure 3.1) and the ANOVA analysis of each group (Table 3.8), Job's tears accessions tested were classified into different groups with significant difference in morphological characteristics. With the genetic distance of 10.5, 94 Job's tears accessions were grouped into seven major clusters.

Table 3.7 Eigen values, proportion of variance, and morphological traits that contributed to the first seven PCs.

Component	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Plant height	-.062	.819	-.040	-.036	-.244	.106	.203
Stem node number	-.183	.769	-.304	.007	.180	-.160	-.114
Number of branches	.467	.006	.079	.324	-.761	.123	-.140
Primer branch nodes	-.234	.689	-.468	-.034	-.092	.086	-.127
Panicle branch number	.304	.445	.538	.162	.179	-.429	-.146
Grain number per plant	.275	.371	.738	.008	-.027	-.016	.172
100-seed weight	.189	-.135	-.390	.786	.077	-.316	.206
Total bract surface characteristics	.934	.132	-.071	.013	.104	.181	.067
Total bract texture	.934	.132	-.071	.013	.104	.181	.067
Total bract shape	.674	.032	-.132	.034	.357	.281	-.028
Total bract color	-.693	.123	.165	.225	.101	.300	.463
Pericarp color	-.515	.089	.289	.496	.214	.411	-.393
Eigen values	3.603	2.150	1.443	1.049	0.922	0.747	0.561
Contribution rate (%)	30.028	17.918	12.032	8.743	7.685	6.227	4.679
Cumulative (%)	30.028	47.946	59.978	68.721	76.406	82.633	87.312

Cluster I contained 21 accessions mainly from Guizhou 7 accessions, Yunnan 6 accessions, Sichuan 3 accessions, Qianxinan Institute 3 accessions, Hebei 1 accessions and Guangxi 1 accessions. Y22, Y85, Y83, Y76, Y88, Y23, Y25, Y17, Y18, Y63, Y74, Y35, Y41, Y51, Y64, Y90, Y58, Y67, Y87, Y73 and Y69 were classified to this cluster .The main features were the plant height which were between 188.8 and 263.4 cm, the stem node number were between 9 and 14.4, the number of branches were between 5.5 and 25.5, the primer branch nodes were between 3.8 and 8.5, the panicle branch number were between 42.2 and 117.0, the grain number per

plant were between 136.0 and 227.8, and the 100-seed weight were between 7.4 and 12.6g .This group was relatively high in plant height, stem node number, number of branches and panicle branch number, but the variance value of panicle branch number was biggest table 3.8

Cluster II contained only Y77. It was a cultivated variety collected from Maguan of Yunan province with the highest plant height 239.8 cm, the most stem node number 11.2 and panicle branch number 120.4, higher100-seed weight 13.4g.

Cluster III contained 4 accessions mainly from Guizhou 1 accession, Yunnan 1 accession, Sichuan 3 accessions, Hebei 1 accession and Chongqing 1 accession. Y11, Y52, Y86, and Y17 were classified to this cluster. The main features were the plant height between 155.4 and 195.0cm, the stem node number were between 8.3 and 12.0, the number of branches were between 6.4 and 7.7, the primer branch nodes were between 1.7 and 4.4, the panicle branch number were between19.9 and 39.5, the grain number per plant were between 237.0 and 266.0, and the 100-seed weight were between 8.1 and 15.2g .This group was relatively low in plant height, number of branches, and primer branch nodes and panicle branch number, but it had relatively high grain number per plant and 100-seed weight.

Cluster IV contained 10 accessions which were mainly from Guizhou 2 accessions, Yunnan 3 accessions, Sichuan 2 accessions, Qianxinan Institute 2 accessions, and Chongqing 1 accessions. This group had the hightest number of branches 11.5 and grain number per plant 270.5, whereas the 100-seed weight 8.8 was lowest.

Cluster Vcontained Y10 and Y18 collected from two different but adjacent counties Changshun and Puding of Guizhou province. This group had the hightest primer branch nodes 6.5. Although this group had lower grain number per

plant, with only 78.9, it was heaviest in the 100-seed weight 5.7g. The variance values of grain number per plant, panicle branch number and stem node number in this group were lowest in all group.

Cluster VI had totally 55 accessions, this group collected form Guizhou 24 accessions, Yunnan 7 accessions, Sichuan 2 accessions, Qianxinan Institute 4 accessions, Hebei 2 accessions, Guangxi 4 accessions, Hunan 7 accessions and Chongqing 5 accessions. All characteristics of this group were in a relatively stable intermediate level, but the traits of grain number per plant and 100-seed weight had the biggest variance values.

Cluster VII contained only Y84. It was a cultivated variety collected from Quejing of Yunnan province with the shortest plant height 59.6cm, least stem node number 7.4, lowest primer branch nodes 1.8, and lowest panicle branch number 25.6 and grain number per plant 55.5. In addition, the number of branches 7.7 and the 100-seed weight 9.1 were also relatively lower.

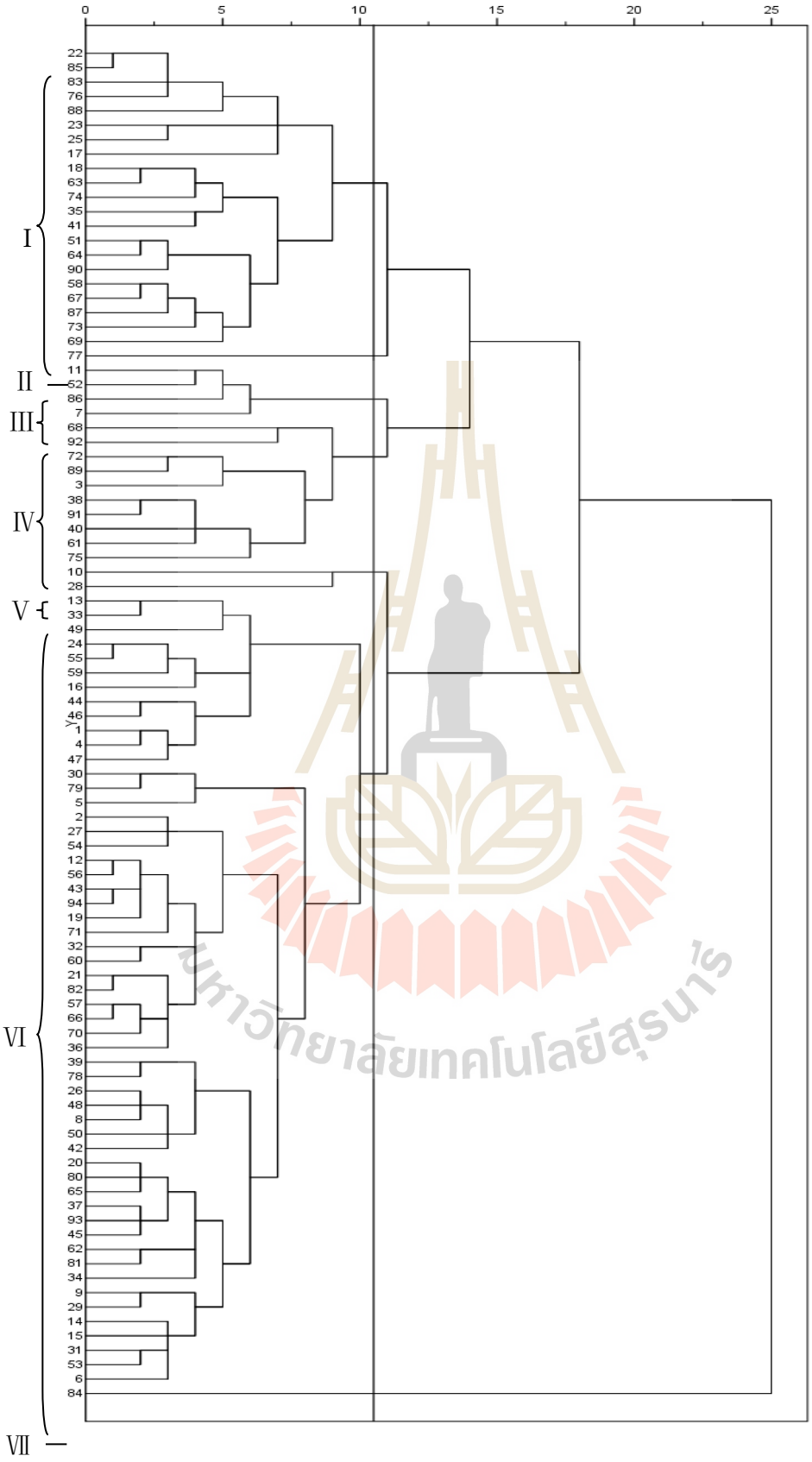

Figure 3.1 The cluster map of 94 accessions based on quantitative and qualitative.

Table 3.8 The ANOVA analyze for traits of each group.

Group		Traits						
		GNP	**NB**	**PB**	**PBN**	**PH**	**SNN**	**SW**
I	Minimum	136	5.5	42.2	3.8	188.8	9	7.4
	Maximum	227.8	25.6	117	8.5	263.4	14.4	12.6
	Mean	187.24	11.043	74.71	5.4238	223.93	11.148	10.105
	SD	21.96	4.8631	21.865	1.352	21.606	1.552	1.7486
	Variance	482.23	23.65	478.09	1.8279	466.82	2.4086	3.0575
II*	Minimum	239.8	11.2	8.4	3.8	120.4	235.8	13.4
	Maximum	239.8	11.2	8.4	3.8	120.4	235.8	13.4
	Mean	239.8	11.2	8.4	3.8	120.4	235.8	13.4
	SD	0	0	0	0	0	0	0
	Variance	0	0	0	0	0	0	0
III	Minimum	237	6.4	19.9	1.7	155.4	8.3	8.1
	Maximum	266	7.7	35	4.7	195	12	15.2
	Mean	250.85	6.95	26.7	3.8	175.52	10.175	10.975
	SD	14.494	0.5568	6.2316	1.4071	18.061	1.565	3.1574
	Variance	210.09	0.31	38.833	1.98	326.18	2.4492	9.9692
IV	Minimum	234	5.7	57.4	2	163.5	234	5.7
	Maximum	316.7	16.7	105.8	6.4	231.5	316.7	16.7
	Mean	270.55	11.53	84.12	3.54	193.23	9.28	8.83
	SD	24.242	4.3336	16.958	1.2394	23.509	1.4359	1.1595
	Variance	587.66	18.78	287.56	1.536	552.67	2.0618	1.3446
V	Minimum	75.8	5	88.4	5	150	10	13.6
	Maximum	76	6.7	100.4	8	214.7	10.7	17.9
	Mean	75.9	5.85	94.4	6.5	182.35	10.35	15.75
	SD	0.1414	1.2021	8.4853	2.1213	45.75	0.495	3.0406
	Variance	0.02	1.445	72	4.5	2093	0.245	9.245
VI	Minimum	45	5	19.9	2.4	146	45	5
	Maximum	159.4	16.4	81.3	8.7	248.4	159.4	16.4
	Mean	106.27	9.1109	45.465	4.5345	195.58	9.9527	10.613
	SD	28.606	2.7871	14.779	1.17	21.807	1.1508	3.2395
	Variance	818.3	7.7677	218.41	1.369	475.53	1.3244	10.494
VII*	Minimum	55.5	7.7	25.6	1.8	59.6	7.4	9.1
	Maximum	55.5	7.7	25.6	1.8	59.6	7.4	9.1
	Mean	55.5	7.7	25.6	1.8	59.6	7.4	9.1
	SD	0	0	0	0	0	0	0
	Variance	0	0	0	0	0	0	0
Cultivate	Minimum	59.2	5.5	25.6	1.7	148.7	6.9	6.7
	Maximum	302.8	25.6	120.4	8.5	263.3	13.4	25.3
	Mean	165.74	11.08	64.407	4.4875	200.91	10.005	10.96
	SD	63.334	4.0289	27.466	1.3502	25.372	1.491	3.2713
	Variance	4011.2	16.232	754.36	1.8232	643.73	2.2231	10.701
Wild	Minimum	45	5	19.9	1.8	59.6	7.4	6.8
	Maximum	316.7	15.7	100.4	8.7	263.4	14.4	19.7
	Mean	135.16	8.5315	51.407	4.6852	198.55	10.261	10.069
	SD	65.75	2.8712	21.085	1.4191	32.412	1.3528	2.5383
	Variance	4323	8.2437	444.58	2.0137	1050.5	1.83	6.443

Note: PH: Plant height, SNN: Stem node number, NB: Number of branches, PBN: Primer branch nodes, PB: Panicle branch number, GNP: Grain number per plant, SW: 100-seed weight. Group II and *VII.consisted of 1 accession *

3.5 Discussion

Germplasm resources collection is an important step in breeding for improvement of the crop (Andini et al., 2013; Nelson et al., 2011). Excavating the excellent germplasm of Job's tears was the most important job for improving the species of Job's tears. The results of this study showed that environment can significant impact the quantitative traits of 94 Job's tears, but the main influence comes from the genetype. Both cultivated and wild accessions have large variation in number of branches, primer branch nodes, panicles per plant and grain number per plant. These accessions have abundant diversity and high range of optional resources for breeding. Cultivated variety Y36 had 25.3g of 100-seed weight more than previous finding 12.5-21.5g (Wang et al., 2013), which could be used as excellent gene resources for the improvement of varieties with a good combination of agronomic materials such as larger number of branches and medium plant height. Y92, Y68, Y3, Y40 and Y72 could be used as the materials for high yield variety with more grain number per plant. Cultivated variety Y30 and wild variety Y84 with lower plant height could be used as excellent gene resources for dwarf new varieties. Y77 had higher plant height, stem node number, number of branches, grain number per plant, 100-seed weight and the hightest panicles per plant. It was an excellent resource with better comprehensive traits.

Grain yield was closely associated with grain number per plant at harvest (Wang et al., 2013). The analysis of simple correlations among the traits revealed that accessions with higher number of branches, plant heighter and stem node number had high grains number in per plant were positive correlated among the accessions suggested that these traits could be used as selection criteria for accessions evaluation.

Panicle branch number was also positively and significantly related to grain number per plant indocated that more panicle branch number the more grain number per plant. The accessions with higher primer branch nodes and 100-seed weight had lower grain number per plant, the more grain number per plant was the lighter the 100-seed weight. The qualitative traits were shown positive or negative correlation to each other.

It is more difficult to analyze the multi-index problems with a number of indicators related to each other. The principal component analysis can simplified multi-index analysis through convertion of original and more related indexes into a new index. Previous studies show that the principal component analysis was an effective method for comprehensive evaluation of crops (Wang et al., 2013). In this study, 12 traits of 94 Coix accessions were reduced to 7 main components by using principal component analysis method with the cumulative contribution rate up to 87.31 %. The first principal component reflected the effective main factor of involucre. The second principal component reflected the effect of the main factor of plant type. The third principal component reflected the effective main factor of yield. The fourth, fifth, sixth and seventh principal component reflected the main factor of the 100-seed weight, the total bract shape, the pericarp color and the total bract color, respectively. Therefore, twelve traits indexes were reduced to seven comprehensive indicators used to represent the original variables, to simplify the data and to reveal the relationship between the variables. They also can provide a favorable scientific basis for the selection of the parents in breeding program (Li et al., 2015). This result also found that the number of branches is one of the most important yield indicators, but it was summarized in the first principal component instead of the second principal component. Therefore, it was necessary to combine the original data and the usage of

dialectical analysis methods in order to remove the apparent phenomenon when we evaluated the quality of germplasm resources by using of principal component analysis. In addition, Breeder must seize the main factors and expand the different traits of breeding materials for accelerate breeding program for new varieties.

Genetic diversity analysis of germplasms using morphological traits is an initial step for crop improvement (Julia et al., 2016; Peratoner et al., 2016; Loumerem et al., 2016). The variations in morphological traits can be used to classify materials into different groups. The shape, color and texture of seeds are important in the classification of Coix species (Schaaffhausen et al., 1952; Rao et al., 2010). Present study combined quantitative and quantitative traits. Ninety-four accessions were grouped into seven clusters. Cluster I contained 21 accessions from Guizhou (7 accessions), Yunnan (6 accessions), Sichuan (3 accessions), Qianxinan Institute (3 accessions), Hebei (1 accession) and Guangxi (1 accession). The accessions in this group had different plant characteristics which are good for breeding material. Cluster II contained only Y77 with the highest in plant height, the most stem node number and panicles per plant, 100-seed weight, and this accession can be used as high-yielding variety and parental material to improved superior dwarfing variety. Cluster III contained 4 accessions which can be used as excellent resources to cultivate dwarfing, anti-lodging and high yield variety. Cluster IV with the hightest number of branches and panicle branch number can be used as excellent parental material to improve crop yield. Cluster V contained only Y10 and Y18 collected from two adjacent place, Changshun and Puding in Guizhou province, indicating that accessions can cluster corresponding with geographical locations. Cluster VI was the biggest group consisting of 55 accessions. Cluster VII contained only Y84 with the shortest plant

height (59.6 cm). It can be used as excellent parent material for cultivated dwarfing and anti-lodging new varieties.

Clustering results showed that the accessions collected from different areas could be grouped together, such as Y11, Y52, Y86 and Y7 collected from Guizhou, Yunnan, Sichuan, Hebei and Chongqing. Some accessions collected form the same area might not be completely grouped together, such as Y35 and Y36 in Cluster I and cluster VI which were collected from Ceheng of Guizhou province. This indicated that genetic differences were not influenced by the geographical differences, and the accessions collected from same area can be very different in genetic variation (Li et al., 2010). Genetic differences were based on the type of variety (wild or cultivated) and qualitative traits with inconsistent with the results of clustering by geographical distribution (Xi et al., 2016). This because of the agronomic traits easily affected by environmental conditions and cultural practices (Wang et al., 2013, Bruschi et al., 2003).

3.6 Conclusion

Morphological diversity analysis in this study showed high variations among the materials according to ANOVA, simple correlations and multivariate analysis. the traits of Job's tears significant impacted by both environment and genetype, but the main influence comes from the genetype. Significant and positive correlation were found between grain number per plant and among other yield-related attributes. Principal component analysis showed that the variations observed were mainly caused by traits such as total bract surface characteristics，total bract texture, total bract shape, plant height, stem node number and primer branch nodes, indicating that their

contribution is important in discriminating the accessions. Cluster analysis grouped the 94 accessions of Job's tears into seven clusters. This indicated high diversity for most of the traits, and demonstrated that genetic differences can not only be based on the geographical differences, type of variety (wild or cultivated) and qualitative traits. Since morphological traits are easily affected by environmental conditions, the genetic relationship cannot be reflected only by the similarity between the morphological characteristics.

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CHAPTER IV

GENETIC DIVERSITY AND POPULATION STRUCTURE OF JOB'S TEARS (*Coix lachryma-jobi* L.) GERMPLASM BASED ON ISSR MARKER

4.1 Abstract

ISSR markers were used to assess the genetic diversity and population structure of 8 populations of Job's tears in China. A genotyping analysis that utilized ten ISSR primer pairs resulted in the production of 116 bands, of which 98 (84.48%) were polymorphic. The Guizhou population (PPB = 81.90%, h = 0.3113, I = 0.4589) was the most genetically diverse, while the lowest genetic diversity was observed in the Hebei population (PPB = 46.55%, h = 0.1842, I = 0.2701). Genetic differentiation analyses including GST analysis and analysis of molecular variance (AMOVA) illustrated that genetic variation was most prevalent within populations while only minor variations were observed among populations. Genetic distance coefficients ranged from 0.0095 to 0.0948 for the 8 populations of Job's tears; the genetic relationship between the Guizhou and Chongqing populations was the closest, while the most distant genetic relationship occurred between the Hubei and Hunan populations. The results of an UPGMA cluster analysis that investigated genetic diversity among the populations were consistent with the genetic distance results. The

results of a STRUCTURE analysis suggested that 94 Job's tears accessions could be grouped into two subpopulations. Moreover, according to a cluster analysis based on the unweighted pair-group method of arithmetic averages (UPGMA) for individuals of Job's tears, accessions were divided into two major clusters. The results of the Bayesian cluster and UPGMA cluster analyses were largely consistent despite minor differences. There was no significant correlation between genetic distance and geographic distance ($r = 0.055$, $p = 0.782$). This study provides us with valuable information pertaining to germplasm collection, genetic improvement, and systematic utilization of Job's tears.

4.2 Introduction

Job's tears (*Coix lachryma-jobi* L.) belongs to the *Coix* genus, the Andropogononeae tribe, and the Gramineae family (Zhou *et al*., 2010). Job's tears, also known as coixseed, tear grass, hato mugi, and adlay, is a tall grain-bearing tropical plant (Taylor, 1953). This crop is predominantly planted in Southeast Asian countries including China, Vietnam, Laos, Japan, and Korea (Diao et al., 2016). Job's tears seeds are sweet to taste and are widely used in both culinary and medicinal practices; they are considered an ideal health food and effective medicine. Indeed, the seeds have been shown to reduce fever, invigorate the spleen, while also exhibiting diuretic, anti-cancer, hypolipidemic, hypoglycemic, antioxidant, anti-inflammatory, and anti-allergic properties (Wen, 2008; Fu et al., 2011, Xi et al., 2016). Job's tears seeds are nutritious and are commonly processed into products including Job's tears

seed tea, Job's tears seed powder, Job's tears seed biscuits, Job's tears seed alcoholic beverages and vinegar (Gu et al., 1999; Liu, 2003.) Until now, research conducted on Job's tears has predominantly focused on agronomic characteristics, inherent chemical constituents and associated pharmacological functions. There is currently a lack of international peer-reviewed articles pertaining to breeding programs associated with Job's tears. Evaluation of genetic diversity and genetic relationships in the germplasm can provide useful information for breeding programs. Morphological or phenotypic descriptors have traditionally been used to distinguish one accession from another. Although this type of agronomical characterization provides useful information, these investigations are subject to environmental influences; they are also time-consuming and must be assessed during a fixed vegetative phase of the crop life cycle (Swanepoel, 1999). Molecular markers have significant advantages over morphological and isozyme markers because they are not influenced by growth and environmental conditions and can be applied during any growth phase. Compared with strategies that analyze other molecular markers, analyses investigating inter-simple sequence repeats (ISSR) are attractive methods because they do not require DNA sequence information. These methods facilitate the detection of highly variable sequences; they are also reproducible and cost effective. ISSR analysis has been widely used to study the genetic diversity of various plants (Rodrigues et al, 2013). Several statistical methods including POPGENE analysis, Analysis of molecular variance (AMOVA), Bayesian cluster analysis and Molecular Evolutionary Genetics Analysis (MEGA) have been developed to analyze population structure, genetic

diversity and differentiation of the germplasm. However, up until now there has been no comprehensive analysis of the genetic diversity and population structure of Job's tears in China. Therefore, a comprehensive analysis of the genetic diversity of Job's tears is required to facilitate the effective utilization of associated accessions during breeding. This current study was undertaken to systematically analyze genetic diversity and population structure in 94 Job's tears accessions using ISSR markers. The purpose of this study was to assess genetic diversity among accessions of Job's tears that were collected from different regions of China and to evaluate the population structure of these accessions.

4.3 Materials and Methods

4.3.1 Plant materials

A total of 94 Job's tears accessions were included in this study (Table 4.1). Forty cultivated accessions and fifty-four wild accessions were sampled from different geographical regions from seven different provinces (Guizhou (36), Guangxi (5), Sichuan (7), Chongqing (7), Hunan (7), Yunnan (19), Hubei (4), and Qianxinan Institute of Agricultural Sciences (9)) of China. All of the Job's tears accessions were selected between 2013 and 2014.

4.3.2 Experiment

Thirty seeds per accession of Job's tears were immersed in 60°C hot water for 30 minutes, and then planted in the seedling cups. Seedlings with 3-4 leaves were transplanted to the field at the Germplasm Resources Garden of Guizhou

Academy of Agricultural Sciences (altitude at 1,074.3 meters above sea level, with latitude and longitude of 106.71 and 26.57, respectively), in Guizhou province of the China, during April 2016. Completely Randomized Design (CRD) with 3 replications was employed. Each replication consisted of 10 plants with 1 plant per hill. The row and plant spacing of each plant were 60 cm and 40 cm, respectively.

4.3.3 Crop management

Phosphate Fertilizer 75,000kg/hectare, Potash Fertilizer 15,000kg/ hectare, Urea 15,000kg/hectare as Base Fertilizer. 45,000 kg/hectare of compound fertilizer was applied after 30 cm in seedling height. Then 2% superphosphate solution was used for extra-root dressing at flowering stage.

Weeding for 2 times. The first weeding was carried out at a plant height of 20 cm; The second weeding was carried out with the fertilization and soil cultivation at a plant height of 30 cm.

Seedling stage, panicle stage, flowering stage and grain filling stage should ensure that there is enough water. In case of drought, it is necessary to water in the early evening to keep the soil moist. After the rain and furrow irrigation, water should be removed. At the same time prevent smut, leaf blight, corn borer and other pests.

4.3.4 DNA extraction

Ninety-four Job's tears accessions (Table 4.1) were studied in this experiment. The leaves of five plants which were randomly selected from 15-day-old seedlings of each accession. These leaf samples were transported to the laboratory in

a 4°C ice box and finally stored in a freezer at -20°C prior to DNA extraction. DNA was extracted into Eppendorf tubes following a modified version of a protocol outlined by Murray and Thompson (1980). Leaf tissues were directly grounded in 500 µL of CTAB (cethyltrimethyl ammonium bromide) extraction buffer containing 2% CTAB, 2 M NaCl, 2% PVP, 20 mM EDTA pH 8.2, 100 mM Tris–HCl pH 8.0, and 1% β-mercaptoethanol (this was performed in Eppendorf tubes). The extracted DNA was quantified on a Microplate Spectrophotometer (Biotic® Instruments, Inc.) and stored at -20°C. Approximately 60 ng of DNA were used in amplification reactions (Dje et al., 2006).

4.3.5 ISSR primer

A total of 100 ISSR primers were screened in this study. The primers were synthesized by Shanghai Sangon Niological Engineering Technology & Service Co., Ltd. China) according to primer sets published by the University of British Columbia (UBC, Canada). Three representative samples from the 94 accessions were initially screened. Ten prime pairs that resulted in the generation of repeatable and polymorphic results with clear bands were used for PCR amplification (Table 4.2).

Table 4.1 The codes and collection place of Job's tears accessions used in this study.

Accession	Collection place	type	Accession	Collection place	type
Y_1	Qianjiang Chongqing	Wild	Y_{48}	Cili Hunan	Wild
Y_2	Qianjiang Chongqing	Wild	Y_{49}	Cili Hunan	Wild
Y_3	Qianjiang Chongqing	Wild	Y_{50}	Cili Hunan	Wild
Y_4	Banan Chongqing	Wild	Y_{51}	Enshi Hubei	Wild
Y_5	Nanchuan Chongqingg	Wild	Y_{52}	Shizong Hubei	Cultivated
Y_6	Nanchuan Chongqing	Cultivated	Y_{53}	Xianfeng Hubei	Wild
Y_7	Wansheng Chongqing	Wild	Y_{54}	Xianfeng Hubei	Wild
Y_8	Changshun Guizhou	Wild	Y_{55}	Longlling Guangxi	Cultivated
Y_9	Changshun Guizhou	Wild	Y_{56}	Longling Guangxi	Wild
Y_{10}	Changshun Guizhou	Wild	Y_{57}	Xilin Guangxi	Cultivated
Y_{11}	Zunyi Guizhou	Wild	Y_{58}	Baise Guangxi	Cultivated
Y_{12}	Huaxi Guizhou	Wild	Y_{59}	Tianlin Guangxi	Cultivated
Y_{13}	Huaxi Guizhou	Wild	Y_{60}	Qianxinan Institute1	Cultivated
Y_{14}	Huaxi Guizhou	Wild	Y_{61}	Qianxinan Institute2	Cultivated
Y_{15}	Huaxi Guizhou	Wild	Y_{62}	Qianxinan Institute3	Cultivated
Y_{16}	Suiyang Guizhou	Wild	Y_{63}	Qianxinan Institute4	Cultivated
Y_{17}	Ziyun Guizhou	Cultivated	Y_{64}	Qianxinan Institute5	Cultivated
Y_{18}	Yuping Guizhou	Wild	Y_{65}	Qianxinan Institute6	Cultivated
Y_{19}	Meitan Guizhou	Wild	Y_{66}	Qianxinan Institute7	Cultivated
Y_{20}	Wangmo Guizhou	Wild	Y_{67}	Qianxinan Institute8	Cultivated
Y_{21}	Wangmo Guizhou	Wild	Y_{68}	Qianxinan Institute9	Cultivated
Y_{22}	Wangmo Guizhou	Wild	Y_{69}	Zizhong Sichuan	Wild
Y_{23}	Wangmo Guizhou	Wild	Y_{70}	Zizhong Sichuan	Wild
Y_{24}	Wangmo Guizhou	Wild	Y_{71}	Jianyang Sichuan	Wild
Y_{25}	Qianlong Guizhou	Cultivated	Y_{72}	Jianyang Sichuan	Wild
Y_{26}	Puding Guizhou	Cultivated	Y_{73}	Jianyang Sichuan	Wild
Y_{27}	Puding Guizhou	Wild	Y_{74}	Chengdu Sichuan	Wild
Y_{28}	Puding Guizhou	Wild	Y_{75}	Tianjin Sichuan	Wild
Y_{29}	Sinan Guizhou	Cultivated	Y_{76}	Maguan Yunnan	Cultivated
Y_{30}	Yinjiang Guizhou	Cultivated	Y_{77}	Maguan Yunnan	Cultivated
Y_{31}	Yinjiang Guizhou	Wild	Y_{78}	Qiubei Yunnan	Cultivated
Y_{32}	Wuchuan Guizhou	Wild	Y_{79}	Xichou Yunnan	Wild
Y_{33}	Wuchuan Guizhou	Wild	Y_{80}	Luoping Yunnan	Cultivated
Y_{34}	Wuchuan Guizhou	Wild	Y_{81}	Luoping Yunnan	Cultivated
Y_{35}	Ceheng Guizhou	Wild	Y_{82}	Luoping Yunnan	Cultivated
Y_{36}	Ceheng Guizhou	Cultivated	Y_{83}	Luoping Yunnan	Cultivated
Y_{37}	Ceheng Guizhou	Cultivated	Y_{84}	Quejing Yunnan	Wild
Y_{38}	Xingren Guizhou	Cultivated	Y_{85}	Wenshan Yunnan	Wild
Y_{39}	Xingren Guizhou	Cultivated	Y_{86}	Wenshan Yunnan	Cultivated
Y_{40}	Xingrenxiashan Guizhou	Cultivated	Y_{87}	Shizong Yunnan	Wild
Y_{41}	Xingrenxiashan Guizhou	Cultivated	Y_{88}	Shizong Yunnan	Cultivated
Y_{42}	Xingyi Guizhou	Cultivated	Y_{89}	Fuyuan Yunnan	Cultivated
Y_{43}	Guanling Guizhou	Cultivated	Y_{90}	Fuyuang Yunnan	Cultivated
Y_{44}	Yongshun Hunan	Wild	Y_{91}	Fuyuan Yunnan	Cultivated
Y_{45}	Sanzhi Hunan	Wild	Y_{92}	Kunming Yunnan	Wild
Y_{46}	Sanzhi Hunan	Wild	Y_{93}	Dali Yunnan	Wild
Y_{47}	Sanzhi Hunan	Wild	Y_{94}	Lijiang Yunnan	Wild

4.3.6 PCR amplification

The ISSR reaction mixture (total volume = 25 μL) contained 2.5 μL of 10 × buffer (0.1 M of Tris–HCl, pH 8.3, 0.5 M KCl), 1 μL of DNA template (50 ng), 2 μL of each primer (10 μM concentration), 0.5 μL of Taq DNA polymerase (2.5 U/μL), 1 μL of $MgCl_2$ (2 mM), 2 μL of dNTPs (2.5 μM) and 16 μL of ddH_2O. The amplifications were performed in an Eppendorf Mastercycler Gradient PCR machine (Eppendorf, Germany) using the following program conditions: an initial denaturation step of 5 min at 94°C; 35 cycles of 94°C for 30 s, 46–60°C (depending on primer pair chosen) for 30 s, 72°C for 30 s; and a final extension at 72°C for 5 min. The amplification products were electrophoresed at 100 V for 45 min on a horizontal gel apparatus (Bio-Rad, SA) using a 1.5% agarose gel in 1 × TAE (pH 8.0). The gels were stained with 0.8 μg/mL ethidium bromide for approximately 30 min, and then photographed under UV light using the UVP-GDS8000 Gel Documentation System (UVP, USA) (Xi et al, 2016).

4.3.7 Data analysis

The discernible and reproducible DNA bands ranging from 250 to 2000 bp were scored 1 for presence and 0 for absence. These bands were used to construct the binary data matrix for statistical analysis. The number of polymorphic bands (NPB), the percentage of polymorphic bands (PPB), Shannon's Information index (I), effective number of alleles (Ne), Nei's gene diversity index (He), genetic differentiation, genetic identity and genetic distance were calculated using PopGene 1.32 (Yeh et al, 1999). Analysis of molecular variance (AMOVA) was used to analyze

genetic diversity using ARLEQUIN version 3.01 (Excoffier and Schneider, 2005). Based on the Nei's genetic distance, cluster analysis was performed to generate a dendrogram; the dendrogram exhibited the genetic relationships among populations and was assimilated using the un-weighted pair-group method with arithmetic mean (UPGMA) using the MEGA 4 v. 4.1 software (Tamura et al., 2007).

The population structure was also estimated using a Bayesian assignment test as implemented using STRUCTURE v. 2.2 software (Pritchard et al., 2000). K (number of populations) values were set from 1 to 16, 6 repetitions. The posterior probabilities were estimated using the Markov Chain Monte Carlo (MCMC) method. Ninety-four Job's tear individuals were clustered using the admixture model program and the genetic clustering number (K) was inferred; burn-in was 100000 and run-length was 1000000. The value of ΔK was calculated from the values of Ln P (D) corresponding to different values of K (Evanno et al., 2005) and the possible genetic structures were analyzed. Accessions were assigned to a subpopulation if the probability of membership was greater than 70% (Liu, 2003). If membership was ≤ 70%, the accessions were assigned to the mixed subpopulation.

The genetic distance matrix was calculated using the NTSYS-pc version 2.1 software package (Rohlf, 2000). The unweighted pair-group method of arithmetic averages (UPGMA) (Sneath, 1973) tree was constructed based on the genetic distance matrix using Molecular Evolutionary Genetics Analysis (MEGA) 4.1 software (Tamura et al. 2007).

Table 4.2 Inter simple sequence repeat ISSR primers and amplification results.

Primer	Sequence (3'-5')	Optimal annealing temperature	Total number of amplified bands	Number of polymorphic bands (NPB)	Percentage of polymorphic loci (PPB)
UBC815	CTC TCT CTC TCT CTC TG	46.9	14	14	100%
UBC818	CAC ACA CAC ACA CAC AG	56.0	13	13	100%
UBC825	ACA CAC ACA CAC ACA CT	52.2	14	12	85.7%
UBC836	AGA GAG AGA GAG AGA GYA	53.9	11	7	63.6%
UBC840	GAG AGA GAG AGA GAG AYT	48.1	14	14	100%
UBC841	GAG AGA GAG AGA GAG AYC	54.7	8	5	62.5%
UBC844	CTC TCT CTC TCT CTC TRC	49.8	7	7	100%
UBC856	ACA CAC ACA CAC ACA CYA	54.7	10	6	60%
UBC857	ACA CAC ACA CAC ACA CYG	56.2	14	11	78.6%
UBC859	TGT GTG TGT GTG TGT GRC	55.2	11	9	81.8%
Total			116	98	84.48%
Average		53.0	11.6	9.8	

Y = (C, T), R = (A, G)

The geographical distance was obtained by the actual latitude and longitude of each population, and the genetic distance was calculated by POPGENE 3.2 software. The Mantel correlation test (Mantel, 1967) in the TFPGA software (Miller, 1997) was used to analyze the correlation between geographical distance and genetic distance for the different populations.

4.4 Results

4.4.1 Polymorphisms and genetic diversity in populations

A total of 100 widespread ISSR primers were tested prior to the genetic diversity analysis. Ten ISSR primers that gave rise to clear, identifiable and polymorphic bands were selected for further anlayses. In total, 116 polymorphic loci were generated from the 10 primers. The 10 primers result in the generation of 116 fragments, of which 98 were polymorphic (84.48%); the percentage range for the polymorphic bands generated from each ISSR primer ranged from 60% to 100%. In each ISSR reaction, the total number of amplified fragments ranged from 7 (UBC-844) to 14 (UBC-815, UBC-825, UBC-840 and UBC-857), with a mean number of 11.6 loci per primer (Table 4.2, Figure 4.1, Figure 4.2, Figure 4.3, and Figure 4.4).

Popgene 1.32 software was used to analyze the genetic diversity index for each population of Job's tears. The main genetic parameters are reported in Table 4.3. Following ISSR analysis, the number of polymorphic bands (NPB) was 98, the percentage of polymorphic bands (PPB) was 84.48%, the observed number of alleles(*Na*) was 1.8448, the effective number of alleles (*Ne*) was 1.5277, the Nei's (1973) gene diversity (*h*) was 0.3049 and the Shannon's information index (*I*) was 0.4525 at the species level.

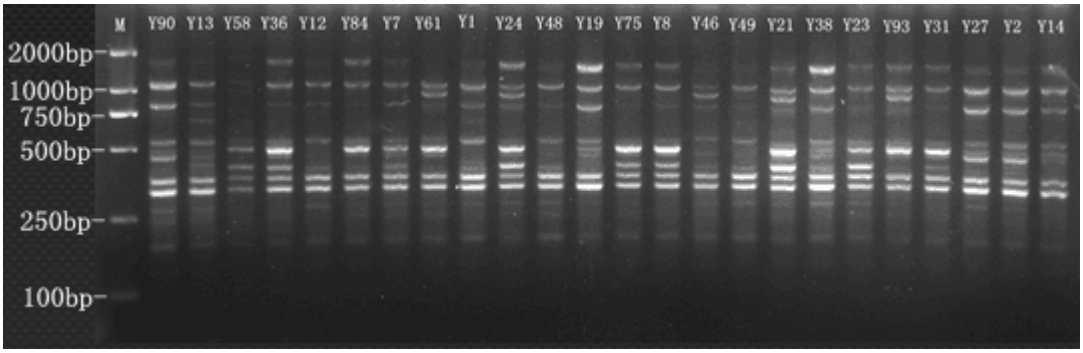

Figure 4.1 Inter simple sequence repeat)ISSR (profiles for 24 Job's tears genotypes following amplification with UBC857 primers and agarose gel eleetrophoresis.

Figure 4.2 Inter simple sequence repeat ISSR profiles for 24 Job's tears genotypes following amplification with UBC857 primers and agarose gel eleetrophoresis.

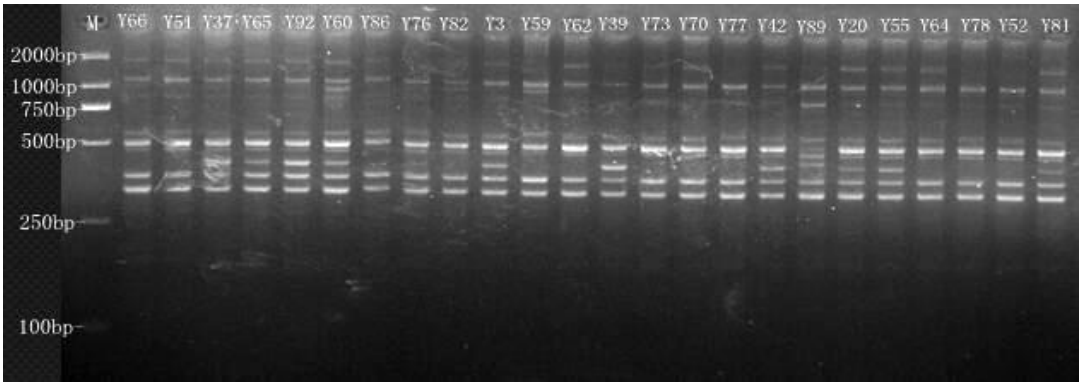

Figure 4.3 Inter simple sequence repeat ISSR profiles for 24 Job's tears genotypes following amplification with UBC857 primers and agarose gel electrophoresis.

Figure 4.4 Inter simple sequence repeat ISSR profiles for 22 Job's tears genotypes following amplification with UBC857 primers and agarose gel electrophoresis.

Table 4.3 Genetic diversity index for 8 populations.

Populations	**Sample Size**	**NPB**	**PPB**	***Na***	***Ne***	***h***	***I***
Chongqing	7	80	68.97%	1.6897	1.4847	0.2727	0.3984
Guizhou	36	95	81.90%	1.8190	1.5412	0.3113	0.4589
Hunan	7	79	68.10%	1.6810	1.4729	0.2663	0.3898
Hubei	4	54	46.55%	1.4655	1.3242	0.1842	0.2701
Guangxi	5	69	59.48%	1.5948	1.4175	0.2342	0.3428
Qianxinan Institute	9	76	65.52%	1.6552	1.4299	0.2434	0.3586
Sichuan	7	70	60.34%	1.6034	1.3977	0.2264	0.3342
Yunnan	19	86	74.14%	1.7414	1.4944	0.2807	0.4130
Average	11.75	76.13	65.63%	1.6559	1.4453	0.2524	0.3706
Species level	94	98	84.48%	1.8448	1.5277	0.3049	0.4525

NPB = Number of polymorphic bands, *PPB* = Percentage of polymorphic bands, *Na* = Observed number of alleles, *Ne* = Effective number of alleles [Kimura and Crow (1964)], *h* = Nei's (1973) gene diversity, *I* = Shannon's Information index [Lewontin (1972)].

The values for each index at the population level were as follows: number of polymorphic bands (NPB) varied from 54 for the Hubei population to 95 for the Guizhou population; the mean value was 76.13. The percentage of polymorphic bands (PPB) varied from 46.55% for the Hubei population to 81.90% for the Guizhou population; the mean value was 65.63%. The observed number of alleles (*Na*) varied from 1.4655 for the Hubei population to 1.8190 for the Guizhou population; the mean value was 1.6559. The effective number of alleles (Ne) varied from 1.3242 for the Hubei population to 1.5412 for the Guizhou population; the mean value was 1.4453. The Nei's (1973) gene diversity (*h*) value varied from 0.1842 for the Hubei population to 0.3113 for the Guizhou population; the mean value was 0.2524. The Shannon's information index (*I*) varied from 0.2701 for the Hubei population to 0.4589 for the Guizhou population; the mean value was 0.3706. The highest genetic diversity was observed in thee Guizhou population (PPB = 81.90%, h = 0.3113, I = 0.4589), whereas the lowest genetic diversity was found in the Hebei population (PPB = 46.55%, h = 0.1842, I = 0.2701).

The eight populations were sequenced according to the detected polymorphic loci, the results for the populations were as follows: Guizhou (NPB = 95, PPB = 81.90%) > Yunnan (NPB = 86, PPB = 74.14%) > Chongqing (NPB = 80, PPB = 68.97%) > Hunan (NPB = 79, PPB = 68.10%) > Qianxinan Institute (NPB = 76, PPB = 65.52%) > Sichuan (NPB = 70, PPB = 60.34%) > Guangxi (NPB = 69, PPB = 59.48%) > Hubei (NPB = 54, PPB = 46.55%).

4.4.2 Genetic differentiation among populations

Genetic differentiation analysis of 8 Job's tears populations revealed that the total gene diversity (Ht) was 0.2950, the gene diversity with provenance (Hs) was 0.2524, the coefficient of gene differentiation (GST) was 0.1443 and the estimate

of gene flow from GST was 2.9654 (Table 4.4). The results revealed that 14.43% of the total genetic variation occurred among populations, while 85.57% of the total genetic variation occurred within populations. These results reveal that genetic variation within populations accounts for the largest proportion of genetic variation. The results also reveal that the estimate of gene flow from GST (Nm) was greater than one (2.9654 > 1); This indicates that the genetic differentiation among populations of Job's tears is not obvious.

To further test the difference between different Job's tear populations we utilized the molecular variance (AMOVA) method. The results revealed that 1.77% of the total variation occurred among the populations, 98.23% of the total variation occurred within the populations, and the variation among the populations and within the populations was significant ($P < 0.05$) (Table 4.5).

The results were similar using both the AMOVA and POPGENE methods. Both methods revealed that there was limited genetic differentiation among the Job's tears populations and the genetic variation predominantly existed within the populations of Job's tears.

Table 4.4 Genetic differentiation among eight populations.

All population	**Size**	**Ht**	**Hs**	**Gst**	**Nm***
Mean	94	0.2950	0.2524	0.1443	2.9654
St. Dev		0.0329	0.0250		

Ht = total gene diversity, Hs = gene diversity with provenance, Gst = coefficient of gene differentiation, *Nm = estimate of gene flow from Gst.

Table 4.5 Results of analysis of molecular variance of ISSR data of Job's tears.

Source of variation	**d.f.**	**Sum of squares**	**Variance components**	**Percentage of variation**	***P***
Among populations	7	138.738	0.29966	1.77	<0.05
Within populations	86	1433.868	16.67288	98.23	<0.05
Total	93	1572.606	16.97254		

4.4.3 Genetic distance and genetic identity

Nei's unbiased measure of genetic distance and genetic identity was utilized to further elucidate the genetic relationship among the Job's tears populations (Table 4.6). The results revealed that the genetic distance ranged from 0.0095 to 0.0948, while the genetic identity ranged from 0.9095 to 0.9905. The Guizhou and Chongqing populations were the least genetically different (0.0095) with a genetic identity value of 0.9905. Conversely, the largest genetic distance (0.0948) occurred between Hubei and Hunan populations with a genetic identity value of 0.9095. These results indicated that the closest genetic relationship occurred between the Guizhou and Chongqing populations while the most distant genetic relationship occurred between the Hubei and Hunan populations. The results also showed that the closest genetic relationship occurred between the Sichuan and Guangxi populations with a genetic distance value of 0.0168 and a genetic identity value of 0.9833. Conversely, the most distant genetic relationship occurred between the Sichuan and Hubei populations with a genetic distance value of 0.0909 and a genetic identity value of 0.9131. The genetic distance between Yunnan and Qianxinan Institute was 0.0173; this indicates that a closer genetic relationship existed between Yunnan and Qianxinan Institute.

Table 4.6 Nei's unbiased Measures of genetic distance (below diagonal) and genetic identity (above diagonal).

Population	Chongqing	Guizhou	Hunan	Hubei	Guangxi	Qianxinan Institute	Sichuan	Yunnan
Chongqing	****	0.9905	0.9681	0.9365	0.9812	0.9612	0.9487	0.9774
Guizhou	0.0095	****	0.9747	0.9447	0.9757	0.9631	0.9687	0.9804
Hunan	0.0324	0.0256	****	0.9095	0.9317	0.9224	0.9227	0.9478
Hubei	0.0656	0.0569	0.0948	****	0.9417	0.9743	0.9131	0.9644
Guangxi	0.0190	0.0246	0.0708	0.0601	****	0.9780	0.9833	0.9742
Qianxinan Institute	0.0395	0.0376	0.0808	0.0261	0.0223	****	0.9454	0.9828
Sichuan	0.0527	0.0318	0.0805	0.0909	0.0168	0.0562	****	0.9545
Yunnan	0.0228	0.0198	0.0536	0.0363	0.0261	0.0173	0.0465	****

4.4.4 Cluster analysis among populations

Relationships between populations were further illustrated by a dendrogram using UPGMA algorithm based on Nei's genetic distance ((Figure 4.5). The illustrated dendrogram grouped the 8 populations into 2 main clusters. Cluster I comprised 5 populations that were further delineated into two sub-clusters. Within cluster I, Guangxi and Sichuan populations constituted one sub-group. Chongqing, Guizhou and Hunan populations clustered into the other sub-group, with Chongqing and Guizhou appearing to be closer to each other than the other populations. The other 3 populations grouped together in cluster II and were further grouped into 2 sub-clusters. Within cluster II, Yunnan and Qianxinan Institute populations constituted one sub-group, while the Hubei population formed a separate sub-group.

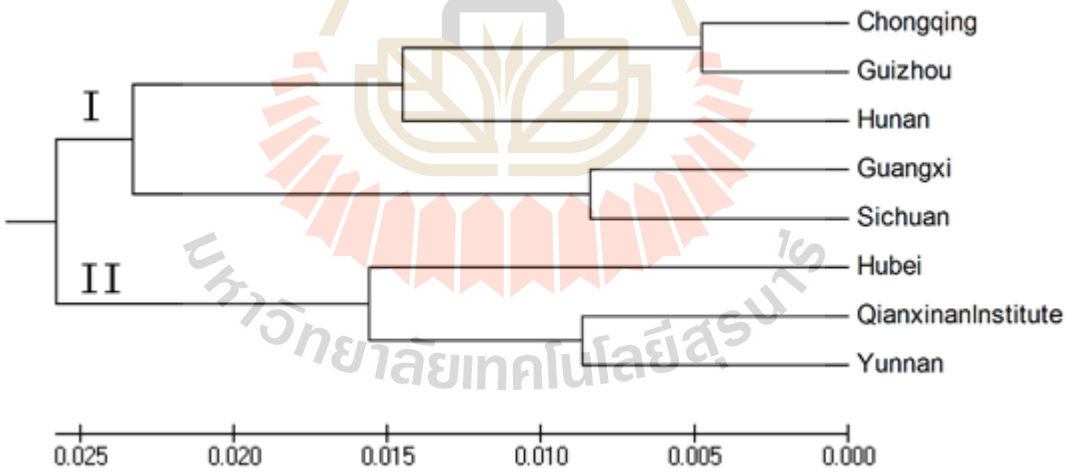

Figure 4.5 Dendrogram of populations of Job's tears generated following UPGMA cluster analysis according to Nei's genetic distance.

4.4.5 Bayesian cluster analysis

STRUCTURE computations do not require prior knowledge of the genetic background of the population; therefore, a STRUCTURE computation analysis was considered an ideal tool for population genetic structure analysis (Falush et al., 2003). The logarithmic rate of change of ΔK can more accurately reflect the true value of K, and the genetic structure of 94 Job's tears individuals were analyzed using the following formula:

$$\Delta K = m[(L(K+1)-2L(K)+L(K-1)]/s[L(K)]$$

In this formula, *L (K)* or *LnP (D)* represents the "*K*" Corresponding logarithm, "*s*" is the standard deviation, and "*m*" is the average. This formula revealed the most suitable number of subgroups to be 2 (Figure 4.6). In total, the 94 accessions of Job's tears can be grouped into 2 subpopulations, G_1 (red) and G_2 (green) (Figure 4.7). Following analysis of membership fractions, the accessions with a probability of > 70%were assigned to corresponding subgroups, while other accessions were categorized as mixed subpopulations (Figure 4.7). Twenty-two accessions (23.40%) were assigned to subpopulation G_1 from Chongqing (3), Guizhou (11), Hunan (2), Guangxi (1), Qianxinan Institute (1), Sichuan (1) and Yunnan (3). Subpopulation G_2 consisted of 61 accessions (64.89%) which were collected from Chongqing (4), Guizhou (20), Hunan (2), Hubei (2), Guangxi (4), Qianxinan Institute (8), Sichuan (6) and Yunnan (15). The mixed subpopulation contained 11 (11.7%) accessions from Guizhou (5), Hunan (3), Hubei (2) and Yunnan (1). From figure 7, we observed that some accessions including Y49, Y23, Y50, Y9, Y28, Y18, Y86, Y54, Y29, Y47 and Y53 shared identity with both the first group (red) and the second group (green).

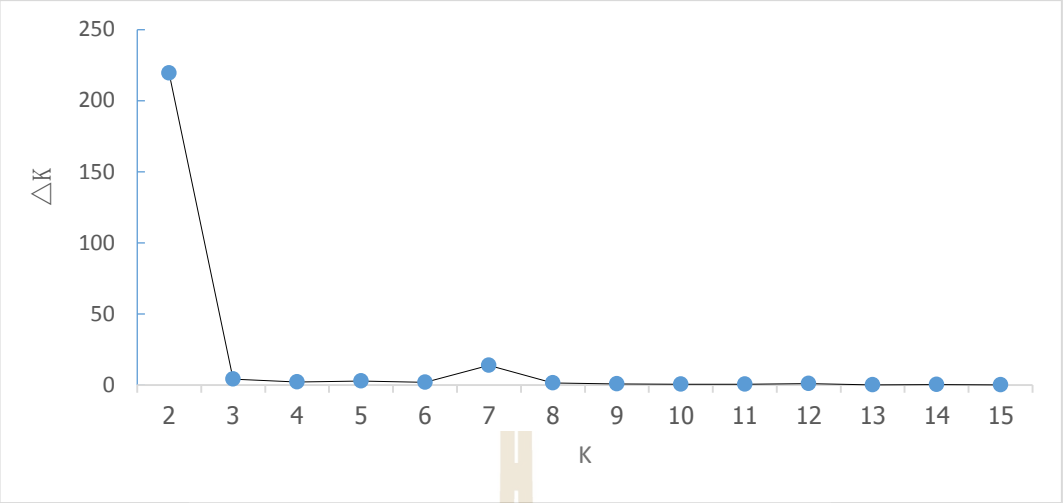

Figure 4.6 The corresponding ΔK values.

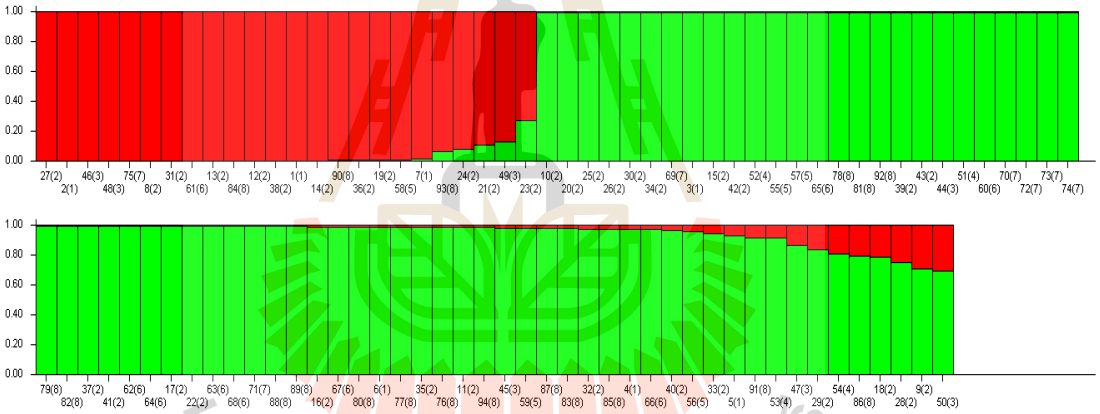

Figure 4.7 Structure analysis for 8 populations of Job's tears where K =2.

Note: 1, 2, 3, ..., 94, represents Y1, Y2, Y3, ..., Y94, respectively. (1), (2), (3), ..., (8) represents Job's tears populations 1–8 (Chongqing, Guizhou, Hunan, Hubei, Guangxi, Qianxinan Institute, Sichuan and Yunnan).

4.4.6 Cluster analysis in individuals accessions

A dendrogram based on the genetic distance matrix of the SRAP data was generated using the UPGMA algorithm (Figure 4.8). In this dendrogram, the 94 accessions of Job's tears were clustered into 2 clusters (Cluster I and Cluster II). The clustering results that were generated according to genetic distance were generally consistent with the results from the STRUCTURE analysis. Cluster I contained 24 accessions of Job's tears: 3 from Chongqing, 12 from Guizhou, 3 from Hunan, one from Guangxi, one from Qianxinan Institute, one from Sichuan and 3 from Yunnan. This group consisted of all of the accessions from group G_1 (Y1, Y2, Y7, Y8, Y12, Y13, Y14, Y19, Y21, Y24, Y27, Y31, Y36, Y38, Y46, Y48, Y58, Y61, Y75, Y84, Y90 and Y93) and 2 accessions from the mixed subpopulation (Y49 and Y23). Cluster II contained 70 accessions and was further divided into 3 subgroups (IIa, IIb and IIc). Subgroup IIa which consisted of IIa_1 and IIa_2 included 24 accessions: 2 from Chongqing, 9 from Guizhou, 2 from Hunan, 2 from Hubei, one from Guangxi, 2 from Qianxinan Institute and 6 from Yunnan. IIa_2 subgroup consisted of 9 accessions from mixed subpopulation (Y18, Y47, Y9, Y28, Y50, Y29, Y86, Y54 and Y53). Subgroup IIb comprised 22 accessions: one from Chongqing, 4 from Guizhou, 2 from Hubei, 2 from Guangxi, 5 from Qianxinan Institute, 2 from Sichuan and 8 from Yunnan.

4.4.7 Correlation analysis of genetic distance and geographical distance

Based on the latitude and longitude of each location, the geographical distance between the populations was measured by GPS, and the genetic distance was calculated using POPGENE3.2 software. Results of the Mantel test revealed no significant correlation between genetic distance and geographical distance ($r = 0.055$; $P > 0.05$) (Figure 4.9).

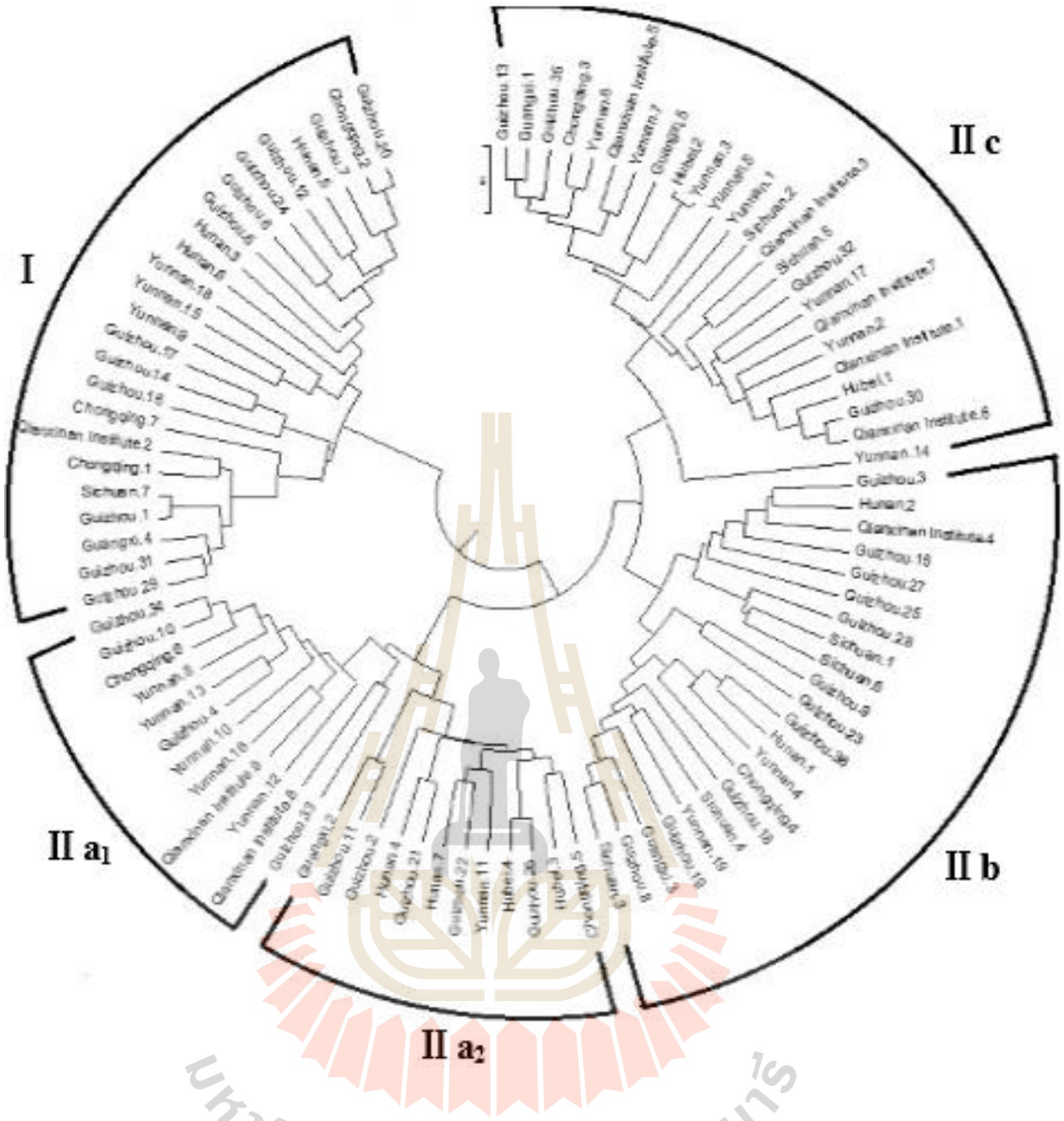

Figure 4.8 UPGMA dendrogram generated from ISSR data showing relationships of 94 accessions of Job's tears.

Note: The code for figure. 8 corresponds to the code for figure 1 (i.e. numbers Y52, Y18, Y47, Y9, Y28, Y50, Y29, Y86, Y54, Y33, Y53 and Y5 for Subgroup II a_2 represent the codes for Guangxi 2 until Hubei3 and Chongqing5).

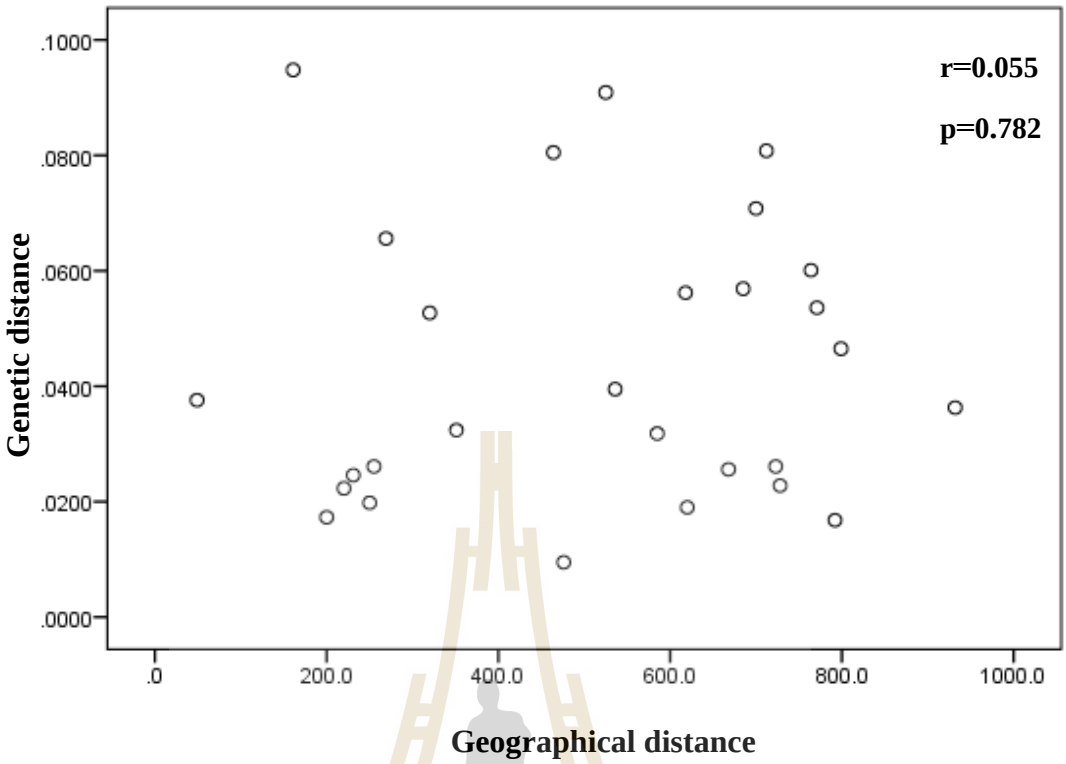

Figure 4.9 MANTEL test plots of the genetic distance and geographical distance.

Note: The vertical axis represents the genetic distance, and the abscissa axis represents the geographical distance.

4.5 Discussion

The genetic diversity of different organisms is predominantly affected by biological characteristics including life-style, breeding methods, eco-environmental factors, related human activities and the evolutionary history of the species. Evolution of a species determines how well a given organism will survive over time. However, the diversity of a population occurs following adaptation to the changing surroundings during the process of evolution; the diversity level can reflect the evolutionary potential of the species (Frankel et al., 1981). In this study, 10 pairs of ISSR primers were selected to analyze the genetic diversity of 8 populations of Job's tears (94

individuals in total). The results showed that 116 loci were amplified using the 10 primer pairs. Of these loci, 98 resulted in polymorphic bands and the polymorphism rate was 84.48%; on average 9.8 polymorphic loci were amplified per primer pair. The ISSR marker is highly polymorphic in Job's tears suggesting that it is more suitable for analyzing the genetic diversity of this plant.

The genetic diversity data of 8 populations in Chongqing, Guizhou, Hunan, Hubei, Guangxi, Qianxinan Institute, Sichuan and Yunnan were analyzed. The results revealed that the percentage of polymorphic loci of the 8 populations varied from 46.55% to 81.90%. The highest percentage of polymorphic loci was observed for the Guizhou population (81.90%), the lowest percentage was observed for the Hubei population (46.55%), and the average percentage of polymorphic loci for the 8 populations was 65.63%. This indicates that the level of genetic variation in Job's tears varies according to geographical location. At the same time, the results also implied that there is reasonably high genetic diversity in Job's tears in the Guizhou population. The latter population also exhibited relatively high levels of adaptability. These observations suggest that the climate in Guizhou is more suitable than other poulations in this study for the growth of Job's tears (Zhou et al., 2011).

At the species level, the observed number of alleles (*Na*) was 1.8448, the effective number of alleles (*Ne*) was 1.5277, Nei's (1973) gene diversity (*h*) was 0.3049 and the Shannon's information index (*I*) was 0.4525; this reveals that the 8 populations of Job's tears exhibit rich genetic diversity. The pattern of genetic diversity in the 8 populations was as follows: Guizhou > Yunnan > Chongqing > Hunan > Qianxinan Institute > Sichuan > Guangxi > Hubei. Thus, the greatest degree of genetic diversity was observed in the Guizhou population, while the Hubei

population had the lowest genetic diversity. There are two possible reasons for this result. First, Southwest China is the origin and main producing area in relation to Job's tears, and associated germplasm resources are richer than those observed in other regions (Gao et al., 2006; Zhou et al., 2006). Secondly, the largest number of samples was collected in Guizhou, while the lowest number was collected in Hubei.

Gene differentiation and gene flow indices are important when evaluating the genetic structure of a species. In the current study, following analysis of the ISSR marker, we observed that the GST value in Job's tears was 0.1443. According to genetic differentiation data, 14.43% of the total genetic variation occurred among the populations and 85.57% of the genetic variation occurred within the populations. This indicates that variation is more prevalent within populations while there are only minor variations among populations. Gene flow, which depicts the gene movement within and between populations, is negatively correlated with gene differentiation (Grant 1991) and is extremely important for population transfer and plant evolution (Hamrick 1987). According to the value of Nm, Govindaraju (1989) divided gene flow into 3 levels: high level ($Nm > 1$), medium level ($0.25 < Nm < 0.99$), and low level ($Nm < 0.25$). Following analysis of the ISSR marker, we observed that gene flow (Nm) was 2.9654 (>1). This indicates that there is no obvious genetic differentiation among populations of Job's tears. This is likely because the spread of Job's tears pollen relies on the wind and the geographic distance between populations (minimum is 49.0 km) is generally significant. These factors hinder genetic exchange between populations, thereby causing inter-population genetic differentiation to be greater than intra-population genetic differentiation.

According to the results of analysis of molecular variance (AMOVA), we observed 98.23% genetic variation within the Job's tears populations, while there was only 1.77% genetic variation among populations. These results are consistent with the genetic differentiation results where intra-population genetic variation was greater than inter-population genetic variation. In fact, this phenomenon was also observed for other species, with Allnutt et al. (2003) using RAPD markers to study diversity in *Pilgerodendron uviferum*; the results showed that 81.4% of the genetic variation occurred within the analyzed populations. Wang et al. (2011) detected 10 populations of *Taihangia rupestris* and observed that 73.51% of the variation existed within the analyzed populations.

The results showed that the genetic distance among the eight populations of Job's tears ranged from 0.0095 to 0.0948, while the genetic identity ranged from 0.9095 to 0.9905. The genetic distance between the Guizhou and Chongqing populations was 0.0095 and the genetic identity was 0.9905, whereas the genetic distance between the Hubei and Hunan populations was 0.0948 and the genetic identity was 0.9095. This indicates that the closest genetic relationship occurred between the Guizhou and Chongqing populations. Conversely, the most distant genetic relationship occurred between the Hubei and Hunan populations. Further, this also shows that there are significant differences in relation to the genetic distance and genetic identity among the 8 populations. The results of an UPGMA cluster analysis showed that the Guizhou and Chongqing populations, the Guangxi and Sichuan populations, and the Yunnan and Qianxinan Institute populations grouped together, respectively. This suggests that there is a close genetic relationship between

populations that group together (Figure 4.5). This is consistent with the genetic distance results.

The Bayesian cluster and UPGMA cluster analyses suggested the existence of two clusters or subpopulations. Despite minor differences, the results were largely consistent. Bayesian cluster analysis can be used to infer genetic structure; this strategy is also used to analyze which populations might be present and to estimate the ancestry of the sampled individuals (Rosenberg NA, 2002). In this study, Bayesian cluster analysis grouped the 94 accessions of Job's tears into 2 groups: G_1 and G_2 (Figure. 7). The dendrogram constructed using the UPGMA clustering algorithm grouped the 94 accessions of Job's tears into 2 clusters; this was in accordance with the results of the Bayesian cluster analysis. All accessions from the G_1 and G_2 subgroups were present in cluster I and II, respectively. Apart from Y23 and Y49, the accessions in cluster I were the same as those in group G_1 (Y1, Y2, Y7, Y8, Y12, Y13, Y14, Y19, Y21, Y24, Y27, Y31, Y36, Y38, Y46, Y48, Y58, Y61, Y75, Y84, Y90 and Y93). The accessions of the mixed subgroup were clustered into 2 clusters (cluster I and cluster II_{a2}) following UPGMA. Two accessions (Y23 and Y49) from the mixed subgroup were clustered into cluster I and 9 accessions (Y18, Y47, Y9, Y28, Y50, Y29, Y86, Y54 and Y53) were clustered into cluster II_{a2} (Figure 4.8). Following comparison with the results generated by the Bayesian cluster analysis, the accessions in the mixed group were not identified in the UPGMA tree. Therefore, Bayesian cluster analysis can not only assign each individual to a hypothetical ancestral cluster(s) without any prior information, but it also reveals admixtures that are not obvious using distance-based clustering methods (Falush et al., 2003).

In general, clustering is carried out according to the location associated with sample collection. However, in this study some samples from the same population clustered in different groups while samples from different populations clustered in the same group. For example, in the Chongqing population, Y1, Y2, Y7 clustered in the first group (red), while Y3, Y4, Y5, Y6 clustered in the second group (green). Y51, Y52, Y57, and Y57 from the Guangxi population and Y62, Y63, Y64, Y65, Y66, Y67, and Y68 from the Qianxinan Institute population clustered in the same group (green).

According to the theory of distance isolation, if there is a balance between gene flow and genetic drift, the genetic distance is positively correlated with the geographical distance (Wright, 1943). When the gene flow rate is very small, genetic drift causes genetic variation (Slatkin, 1997; Hutchison, 1999). In the present study, we did not observe a correlation between genetic distance and geographical distance ($r = 0.055$, $P = 0.782$). This suggests that gene flow and genetic drift among populations were not balanced, and that genetic drift plays a greater role in population structure than gene flow.

4.6 Conclusion

1. The 10 pairs of ISSR primers that were used in this screening study resulted in the amplification of 98 polymorphic DNA fragments (84.48%) with an average of 9.8 polymorphic bands per primer. This indicates that the selected primers can be used to assess polymorphism levels and are suitable for genetic diversity analysis for Job's tears.

2. Analysis of genetic diversity and genetic structure of 8 populations of Job's tears. The results showed that there is a high level of genetic diversity in the Job's

tears populations that were analyzed in this study. The Guizhou population exhibited the highest genetic diversity, while the Hubei population had the lowest genetic diversity.

3. Both the genetic distance and UPGMA cluster analyses for the 8 Job's tears populations revealed that there was a close genetic relationship between the Guizhou and Chongqing, the Guangxi and Sichuan, and the Yunnan and Qianxinan Institute populations, respectively.

4. In the current study, we observed following ISSR marker analysis that the GST value in Job's tears was 0.1443; this indicated that 14.43% of the total genetic variation occurred among the populations, while 85.57% of the genetic variation occurred within the populations. The results of an analysis of molecular variance (AMOVA) showed that 98.23% of the genetic variation occurred within the populations, while the genetic variation among populations was only 1.77%. Both GST and AMOVA results revealed that total variation was most prevalent within Job's tears populations while only minor variations existed among populations.

5. Bayesian cluster and UPGMA cluster analyses produced similar results in relation to the Job's populations. Ninety-four Job's tears accessions were grouped into 2 clusters. Cluster I contained 24 Job's tears accessions: 3 from Chongqing, 12 from Guizhou, 3 from Hunan, one from Guangxi, one from Qianxinan Institute, one from Sichuan and 3 from Yunnan. Cluster II contained 70 accessions: 4 from Chongqing, 24 from Guizhou, 4 from Hunan, 4 from Guangxi, 4 from Guangxi, 8 from Qianxinan Institute, 6 from Sichuan and 16 from Yunnan. At the same time, the results show that accessions predominantly clustered in accordance with the collection areas analyzed.

6. The results of the Mantel test showed that there was no correlation between geographic distance and genetic distance among the populations of Job's tears (r = 0.055, p = 0.782). We also observed that genetic drift plays a greater role in population structure than gene flow.

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CHAPTER V

INDUCED MUTATION OF JOB'S TEARS (*Coix lachryma-jobi* L.) BY ETHYL METHANESULFONATE AND GARMMA RADIATION

5.1 Abstract

Induced mutations are highly effective in enhancing genetic variation of crops, and have been successfully employed in developing improved cultivars. The technique might be used in Job's tears breeding program to alternate the gene by a vaieties of mutagent.

The aim of present study was to explore the mutagenesis of Ethyl methanesulfonate (EMS) and ^{60}Co-γ radiation treated Job's tears cultivars and to determine the possibility of different mutagent in breeding programe**.** Two Job**'**s tears accessions Y159 collected from Luoping of Yunnan province of China and CDT collected from Thailand were used in this experiment**.** The seeds of both cultivated varieties Y159 and CDT were treated by different dose of ^{60}Co-γ radiation and concentrations of EMS. The results showed that different dose of ^{60}Co-γ radiation and concentrations of EMS had a significant impact on seed germination, seedling heights and mutation rate. The LD50 of ^{60}Co-γ radiation irradiated for CDT and Y159 were 406.305 and 284.795 Gy, respectively, the LD50 of EMS treated for Y159 was 2.453%

in concentration. The results of genetic diversity showed that Job's tears mutant has large variation and rich genetic diversity after mutagenesis treated. Cluster analysis revealed that the 265 gamma-irradiated samples were divided into three groups, the samples in Group I had a higher degree of genetic variation than Group II and Group III, respectively. Cluster analysis also showed that the 204 samples treated with different concentrations of EMS were divided into three groups. The samples in Group I and Group II had higher genetic variation than Group III. These results suggested that ^{60}Co-γ radiation and EMS can be in Job's tears breeding program.

5.2 Introduction

Mutation breeding is purposeful method in plant breeding in order to generate mutants with desirable traits of crops. This has been widely used as a potent method of enhancing variability for crop improvement (Singh and Singh, 2001). Induced mutations are highly effective in enhancing natural genetic resources, and have been successfully employed in developing improved cultivars of cereals, fruits, and ornamentals (Lee et al., 2002; Mohamad et al., 2005). The technology is simple, relatively cheap to perform and equally usable on a small and large scale (Siddiqui and Khan, 1999). By varying the mutagenic agent dose, the frequency and saturation of mutations can be regulated (Menda et al., 2004). Mutagenic agents can induce different extensions of genomic lesions, ranging from base mutations to larger fragment insertions or deletions (Kim et al., 2006). Induced mutation, using physical and

chemical mutagen, is a way to generate genetic variation, resulting in the creation of new varieties with better characteristic (Wongpiyasatid, 2000). In a little less than a century, mutation breeding programs resulted in developing more than 3200 crop varieties that are being grown all over the world (FAO/IAEA, 2012).

Gamma rays and EMS might be applied to develop new varieties with high yield and other improved organic traits (Khatri et al., 2005). Gamma rays are the most energetic form of electromagnetic radiation, their energy level is from ten to several hundred kilo electron volts and they are considered as the most penetrating compared to other radiations (Kovacs and Keresztes 2002). Gamma radiation can be useful for the alteration of physiological characters (Kiong et al., 2008). It can damage or change important components of plant cells. They have been reported to affect differentially the morphology, anatomy, biochemistry and physiology of plants depending on the radiation dose (Ashraf et al., 2003). Ethyl Methane Sulphonate (EMS), also be used as mutagent, is mutagenic and carcinogenic organic compound**.** It produces random mutations in genetic material by nucleotide substitution; particularly by guanine alkylation and it is reported to be the most effective and powerful mutagen (Hajara, 1979). EMS usually causes point mutations in crops (Okagaki et al., 1991). It induces a high rate of mutations in both micro and higher organisms, and sometimes the mutation frequencies more than those obtained by radiation (Freese et al., 1963). John et al., (1999) found that EMS have more effective and efficient than physical mutagens in crops like ocowpea (John et al., 1999).

In the previous study, identification of improved new mutants has been based on morphological characters, but development of DNA techniques has made it faster and precise (Atak et al., 2004). Therefore, the PCR-based molecular marker techniques such as randomly amplified polymorphic DNA **(**RAPD**)**, amplification fragment length polymorphism (AFLP) and ISSR (Inter-Simple Sequence Repeats) have been used for the genetic characterization of mutant plants (Lu et al., 2007; Khatri et al**.**, 2011; Mudibu et al., 2011). Compared to other molecular markers, ISSR is easy to apply, highly informative, reliable, repeatable and inexpensive (Reddy et al., 2002; Semagn et al**.**, 2006). However, there are lack of information on induced mutation in Job's tears (*Coix lacryma-jobi* L.), the high potential food and alternative medicine crop.

Hence, the aim of this study were to explor the mutagenesis and DNA marker technology of Job's tears, and to investigate suitable mutagenic dose for mutation breeding**.** After the seeds were treated with gamma rays and EMS，ISSR analysis were used for detection of genetic differences**.** This will provide theoretical basis and technical support for the mutation breeding of Job's tears.

5.3 Materials and Methods

5.3.1 Plant materials

Two Job's tears accessions Y159 with high yield and high disease resistance collected from Yunnan province of China, and CDT with high temperature and drought resistance collected from Thailand were used in this experiment.

5.3.2 Experiment

The seeds of each accessions was evenly divided to seven dilly bags. Seeds were exposed to seven doses of ^{60}Co-γ ray irradiation (150, 250, 350, 450, 550, 650, and 750 GY). Untreated (0 Gy) was used as a control. The seeds of Y159 accessions were treated with Ethyl methane sulfonate (EMS). Shelled Job's tears seeds were divided into thirteen dilly bags, and put into the container containing different concentrations of EMS (0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6, 4.0, 4.4, 4.8 and 5.2%) for ten hours. Each treatment consisted of 100 seeds with 3 replications. Without EMS treatment was used as control. After ten hour, the shelled seeds were taken out from EMS solution and rinsed five times with sterile distilled water to avoid over treated and EMS residual.

5.3.3 Crop management

After treated with ^{60}Co-γ radiation and EMS, each individual seed was planted in a seed tray filled with peat moss with 1 cm of seed depth. The seed tray was with 6 cm deep and diameter of 4 cm each hole. Seed trays were be put in greenhouse with temperature and humidity of 26℃ and 80% RH, and then watered every 2 days.

5.3.4 Data collection

Germination rate of each accession were calculated form 12 days after sowing according to the formula : germination rate= (germination number/total number of seeds) × 100, and the LD_{50} were calculated based on the germination rate. Seedling height were measured (Measurement the length of seedling from base to apex tip of the

plant) after the seedling have 3-4 leaves (25days days after sowing). The mutation rate was be calculated (mutation rate = mutation plants number/total plants×100) by compairing with the characteristic of the control plant. The leaf of single plant from each treatment were sampled for the genetic diversity analyzed by using ISSR marker. The DNA extraction, PCR amplification and data collection were the same as described 4.3.4 and 4.3.6 of chapter IV.

5.3.5 Data analysis

The unweighted pair-group method of arithmetic averages (UPGMA) (Sneath RR, 1973.) tree was constructed based on the genetic distance matrix by using the Molecular Evolutionary Genetics Analysis (MEGA) 4.1 software (Tamura et al. 2007).

5.4 Results

5.4.1 Different ^{60}Co-γ radiation dose effected on seed germination

Seeds began to germinate from 4 days after sowing untill 12 days after sowing. The germination was delayed when the dose of radiation increase, the effect was obvious when the dose of radiation exceeded 350 Gy. Low-dose radiation was able to increase seed germination rate. On the fourth and fifth days, the germination rates of 150 and 250 Gy-irradiated seeds were significantly higher than that of the control, and the effect varied among the Job's tears cultivars (Table 5.1).

Table 5.1 Different irradiation dose effected on seed germination (%).

Irradiation dose(Gy)	CDT					Y159				
	4d	5d	6d	8d	12d	4d	5d	6d	8d	12d
0	3	35.3	78.3	93.3	95	0	0	8.3	72.3	74.7
150	27.7	66.3	72.7	82.3	84.7	1.7	5.7	20.3	34.3	75.0
250	0	27.3	56.7	82.3	92	0	7.3	29.3	44.0	67.7
350	0	4.3	19.3	44.7	62.7	0	1.0	5.3	23.3	43.3
450	0	4.0	17.7	32.7	33.7	0	0	3	4.7	6.0
550	0	0.6	14.3	22.0	23.7	0	0	0	1.7	1.7
650	0	2.0	6.0	13.0	13.0	0	0	0	0	0
750	0	0	1.3	7.7	10.7	0	0	0	0	0

The germination rates of control seeds and 250 Gy-irradiated seeds were significantly higher than those of other treatments in CDT. For Y159, the germination rates of control seeds and 150 Gy-irradiated seeds were significantly higher than those of other treatments (Table 5.2).

All doses of radiation had a significant impact on seed germination. Germination rate gradually decreased with increasing irradiation dose. The effect of radiation on germination varied among the Job's tears cultivars, and it was most obvious for Y159, as almost of its seeds failed to germinate when the radiation dose exceeded 450 Gy. The effect of radiation on CDT was less, 10.7% still germinated when the radiation dose reached 750 Gy. Hence, radiation at 150-450 Gy is suitable for

CDT and Y159 according to their germination rate at different radiation doses measured above.

5.4.2 Different radiation dose effected on seedling growth

Mutation rate (Leaf abnormal) and seedling height were measured at 25 days after seed sowing. The results showed that mutation rates of two Job's tears cultivars, CDT and Y159, increased with increasing irradiation dose, and reached the maximum level at 450 Gy which was significantly higher than that of other groups (Table 5.3). Y159 seeds failed to germinate when the radiation dose exceeded 650 Gy. Hence, there was no data of mutation rate in these treatments. The seedling heights of two cultivars gradually decreased with increasing radiation dose. The seedlings of two cultivars exposed to more than 550 Gy of radiation all died, so there was no data for seedling height in these treatments.

The plants exposed to radiation showing different leaf shape from control plants were considered to be mutants. The mutation rate of CDT and Y159 increased with increasing radiation dose. Mutation rate was little affected by radiation at low doses, but dramatically increased with the increasing of radiation dose from 250 Gy, and reached almost 100% at radiation dose of 450 Gy.

Table 5.2 Different 60Co-γ radiation dose effected on seed germination of CDT and Y159 cultivars.

Irradiation Dose (Gy)	No. of samples	CDT		Y159	
		No. of germination	Germinaion rate (%)	No. of germination	Germination rate (%)
Ck	300	285	95.0a	224	74.7a
150	300	254	84.7 b	223	74.3a
250	300	276	92.0 a	203	67.7b
350	300	188	62.7c	130	43.3c
450	300	101	33.7d	18	6.0d
550	300	71	23.7e	5	1.7de
650	300	39	13.0f	0	0e
750	300	32	10.7f	0	0e

Table 5.3 Different 60Co-γ radiation dose effected on leaf mutation rate and seedling height.

Irradiation	CDT					Y159				
Dose (Gy)	No. of germination	No. Of abnormal	Abnormal rate (%)	Seedling Height (cm)	Height amplit-ude	No. of germi-nation	No. of abnor-mal	Abnormal rate (%)	Seedling Height (cm)	Height amplitude
Ck	285	0	0d	8.8a	6.5-10.0	224	0	0e	7.2a	3.0-9.0
150	254	22	8.6c	7.5b	2.2-10.0	223	61	27.3d	5.7b	1.0-8.0
250	276	169	61.3b	4.3c	1.0-8.0	203	109	53.4c	3.7c	1.0-10.0
350	188	140	67.5b	2.2d	0.5-12.0	130	125	81.0b	1.2d	0.5-2.5
450	101	94	93.1a	0. 8e	0.5-1.0	18	18	100.0a	1.0d	0.3-2.5
550	71	71	100.0a	-	-	5	5	100.0a	-	-
650	39	39	100.0a	-	-	0	-	-	-	-
750	32	32	100.0a	-	-	0	-	-	-	-

Growth of seedlings was sensitive to radiation and the dose of radiation significantly affected growth rate of the seedlings. Radiation above 150 Gy significantly inhibited growth of seedlings in a dose-dependent manner. And the seedlings exposed to radiation above 450 Gy stopped growing. The influence of radiation dose was nearly the same between the two cultivars. However, CDT cultivars seem to be more tolerance to radiation than Y159.

5.4.3 Different EMS concentrations effected on seed germination and growth

There were no significant differences in germination rate, mutation rate (Leaf abnormal rate) and seedling height among treatments with less than 1.2% of EMS concentration, but the germination rate of these treatments was significantly higher, and the mutation rate was significantly lower than those of other treatments. The concentration of EMS more than 4.4% had no significant effect on germination rate and mutation rate, but a significant effect on seedling height (Table 5.4).

Seed germination rate was significantly affected by the concentration of EMS. Germination gradually decreased with increasing EMS concentration. Germination rate was slightly affected by EMS concentration lower than 1.2%, but significantly affected by EMS concentration higher than 1.2%. Germination rate of Y159 seeds treated with EMS at concentrations greater than 4.4% was close to zero. In addition, the mutation rate was also greatly affected by the concentration of EMS, it gradually increased with increasing EMS concentration. EMS at low concentrations showed no significant

influence on mutation rate, but higher concentrations over 1.2% significantly affected the mutation rate, which increased with increasing EMS concentration. The mutation rate of 4.0% EMS-treated Y159 seeds was up to 100%.

Growth of seedlings were sensitive to EMS. The growth rate was affected by EMS at concentrations of more than 0.4%, and significantly decreased when EMS concentration exceeded 1.2%. However, the inhibition was slightly alleviated at 2.0, 2.4, 3.2, 3.6 and 4.0%, respectively indicating that the effect of EMS on seedling height was not completely determined by EMS concentration.

5.4.4 Different mutagenesis treatments effected on LD50

The median lethal dose (LD50) value was determined based on the seed germination percentage (Anbarasan et al., 2013) using the Statistical Package for Social Science (SPSS 20.0 Inc., USA software) (Akçay A., 2013). The analysis revealed that the regression equations for gamma-irradiated CDT and Y159 were; Probit= 2.222-0.005X and Probit= 2.053-0.007X, and their LD50 were 406.305 and 284.795 Gy, respectively. The regression equation for EMS-treated Y159 was Probit= 1.807-0.737X, and the LD50 was 2.453%. The data points in each scatter plot in figures 5.6, 5.7 and 5.8 fell on or very near a straight line, indicating that the probit models fit the data well.

Table 5.4 Different EMS concentrations effected on seed germination, leaf abnormal rate and seedling height.

EMS concent-rations (%)	No. of samples	No. of germination	Germination rate (%)	No. of abnormal	Abnormal rate (%)	Seedling Height (cm)	Height amplitude (cm)
Ck	300	253	84.3a	0d	0g	6.8a	5.5-8.3
0.4	300	253	84.3a	0d	0g	5.6ab	1.1-8.6
0.8	300	250	83.3a	3	1.2g	5.4ab	1.2-9.3
1.2	300	246	82.0a	24	9.7fg	4.8abc	1.8-7.5
1.6	300	223	74.3b	31	13.9f	4.0bcd	1.1-7.2
2.0	300	215	71. 7b	61	28.5e	4.9abc	0.8-7.2
2.4	300	175	58.3c	77	44.0d	4.8abc	1.2-8.5
2.8	300	155	51.7d	92	60.1c	4,0bcd	1.3-7.1
3.2	300	123	41.0e	104	64.6a	5.5ab	2.2-8.1
3.6	300	61	20.3f	58	95.2a	5.4ab	0.7-9.4
4.0	300	20	6.7g	20	100a	5.4ab	5.2-5.5
4.4	300	5	1.7h	5	100a	3.8bcd	0.5-6.1
4.8	300	2	0.7h	2	100a	2.7cd	1-4.4
5.2	300	1	0.3h	1	100a	2.4d	0.5-4.2

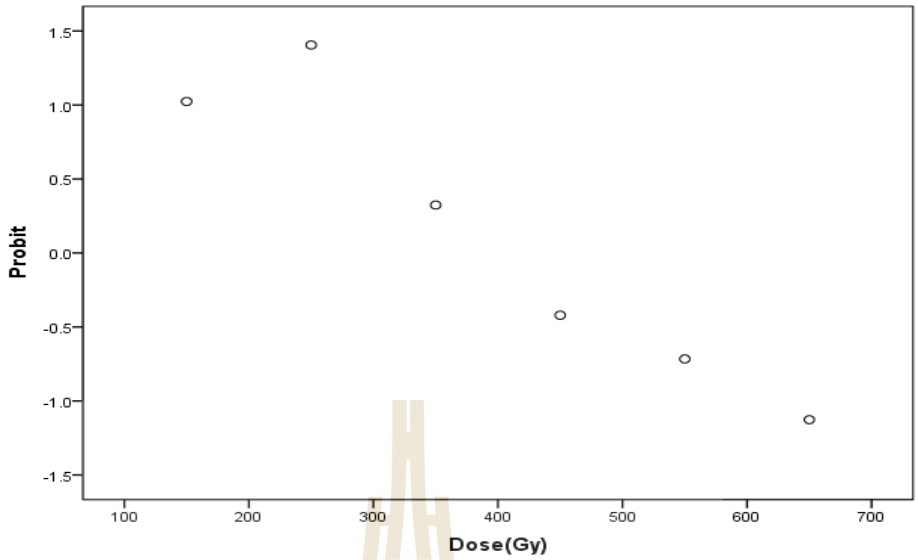

Figure 5.1 60Co-γ radiation dose -response scatter plot of CDT variety.

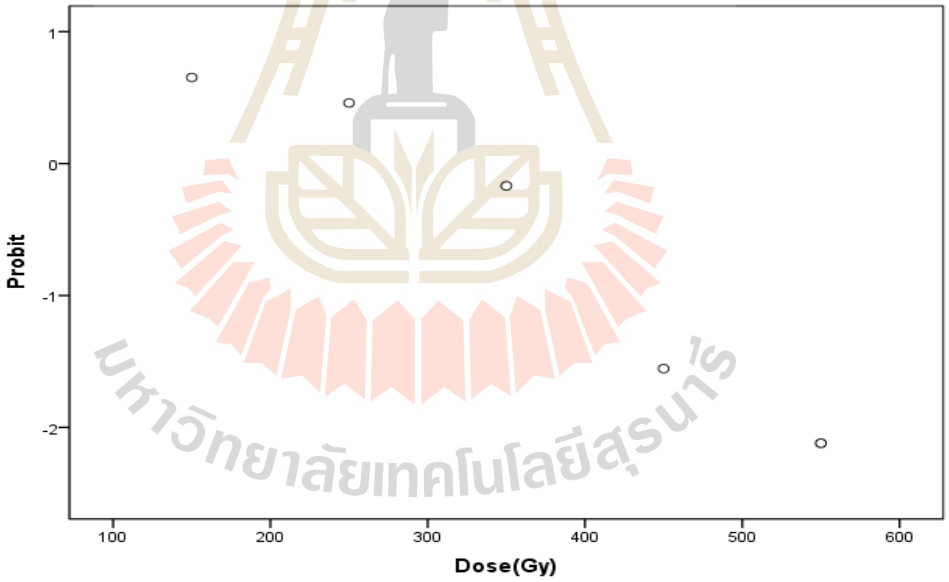

Figure 5.2 ^{60}Co-γ radiation dose - response scatter plot of Y159 variety.

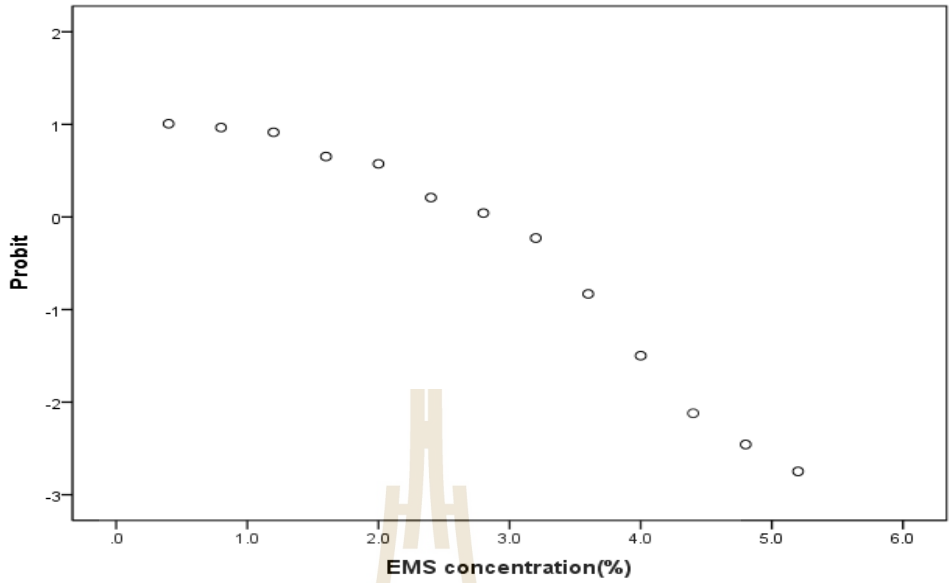

Figure 5.3 EMS concentriations -response scatter plot of Y159 variety.

5.4.5 Genetic diversity of 60Co-γ -irradiated Y159 based on ISSR markers

5.4.5.1 Polymorphism and genetic diversity

Based on the LD50 value of gamma irradiation for Y159 (284.795 Gy) obtained above, Y159 seeds were exposed to 0 (control), 150, 200, 250, 300 and 350 Gy of gamma ray before sowing in this experiment. The leaves of three-to four-leaf seedlings were sampled at random and subjected to ISSR analysis for assessment of genetic diversity. Total of 265 samples were collected, including 10 samples exposed to 0 Gy (control), 60 samples exposed to 150 Gy, 49 samples exposed to 200 Gy, 53 samples exposed to 250 Gy, 72 samples exposed to 300 Gy and

21 samples exposed to 350 Gy. Five of the ten primers used in chapter IV were selected for ISSR analysis (Table 5.5).

As a result, a total of 57 DNA fragments were amplified from the 265 gamma-irradiated samples using the five primers, and 34 of the DNA fragments were polymorphic (59.6%). Nine to fourteen DNA bands were amplified with each primer with an average of 11.4 bands per primer. Four to nine of the DNA bands amplified with each primer were polymorphic, with an average of 6.8 polymorphic bands per primer. Nine polymorphic bands were produced with either UBC836 or UBC857, and the percentages of polymorphism were 81.8% and 64.3%, respectively. Only four polymorphic bands were generated with UBC818, and the percentage of polymorphism was 30.8% (Table 5.5, Figure 5.4 and Figure 5.5).

5.4.5.2 Cluster analysis

Cluster analysis revealed that the 265 gamma-irradiated Y159 samples were divided into three groups (Figure 5.6). Group I was composed of six samples 200-7, 200-49, 250-21, 300-7, 300-8 and 300-12, all of which had been exposed to 200-300 Gy of gamma ray, accounting for 2.3% of all samples (Figure 5.6-I). There were nine samples in Group II (accounting for 3.4% of all samples), including four 150 Gy-irradiated samples, three 250 Gy-irradiated samples and two 350 Gy- irradiated samples (Figure 5.6-II).

Table 5.5 Inter simple sequence repeat primers and amplification results by using 60Co-γ radiation.

Primer	**Sequence (3' –5')**	**Optimal annealing temperature**	**Number of total amplified bands**	**Number of polymorphic bands (NPB)**	**Percentage of polymorphic loci(PPB)**
UBC818	CAC ACA CAC ACA CAC AG	56.0	13	4	30.8%
UBC836	AGA GAG AGA GAG AGA GYA	53.9	11	9	81.8%
UBC841	GAG AGA GAG AGA GAG AYC	54.7	9	7	77.8%
UBC856	ACA CAC ACA CAC ACA CYA	54.7	10	5	50.0%
UBC857	ACA CAC ACA CAC ACA CYG	56.2	14	9	64.3%
Total			57	34	59.6%
Average		55.1	11.4	6.8	

Y=(C, T), R= (A, G)

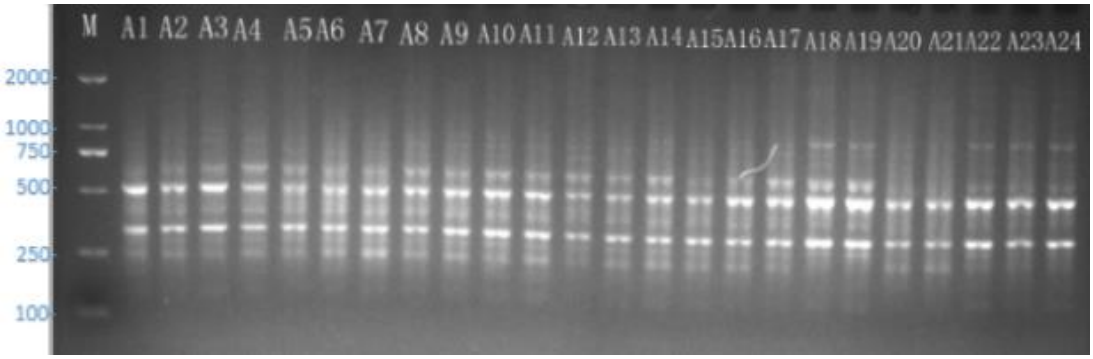

Figure 5.4 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: A1 to A24 represents 150 Gy irradiated 1 to 24 samples number.

Figure 5.5 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836 .

Note: A25 to A48 represents 150 Gy irradiated 25 to 48 samples number.

Group III consisted of 250 samples, accounting for 94.3% of all the samples. All samples of control were included in this group. Group III was further divided into three subgroups. Subgroup IIIa was composed of 76 samples

(Figure 5.6-IIIa), including 21.7% of the 150 Gy-irradiated samples, 38.8% of the 200 Gy-irradiated samples, 24.5% of the 250 Gy-irradiated samples, 36.9% of the 300 Gy-irradiated samples, and 9.5% of the 350 Gy-irradiated samples, and two samples of the control. Subgroup IIIb consisted of 66 samples, including 50.0% of the 150 Gy - irradiated samples, 33.3% of the 350 Gy-irradiated samples, and four samples of the control (Figure 5.6-IIIb). Subgroup IIIc consisted of 108 samples, including 47.1% of the 250 Gy-treated samples, 46.6% of the 300 Gy-treated samples, 47.6% of the 350 Gy-treated samples and 4 samples of the control (Figure 5.6-IIIc).

5.4.6 Genetic diversity of EMS treated Y159 based on ISSR markers

5.4.6.1 Polymorphism and genetic diversity

Y159 seeds were treated with different concentrations of EMS before sowing. The leaves of three-to four-leaf seedlings were sampled at random and subjected to ISSR analysis for assessment of genetic diversity. Total of 204 samples were collected, including 22 samples treated with 0.4% EMS, 26 samples treated with 0.8% EMS, 53 samples treated with 1.2% EMS, 14 samples treated with 1.6% EMS, 20 samples treated with 2.0% EMS, 17 samples treated with 2.4% EMS, 26 samples treated with 2.8% EMS, 30 samples treated with 3.2% EMS, 8 samples treated with 3.6% EMS, 3 samples treated with 4.0% EMS, 2 samples treated with 4.4% EMS, one sample treated with 4.8% EMS, one sample treated with 5.2% EMS, and 10 samples treated with 0% EMS (control). Five of the ten primers used in chapter IV were selected for ISSR analysis (Table 5.6).

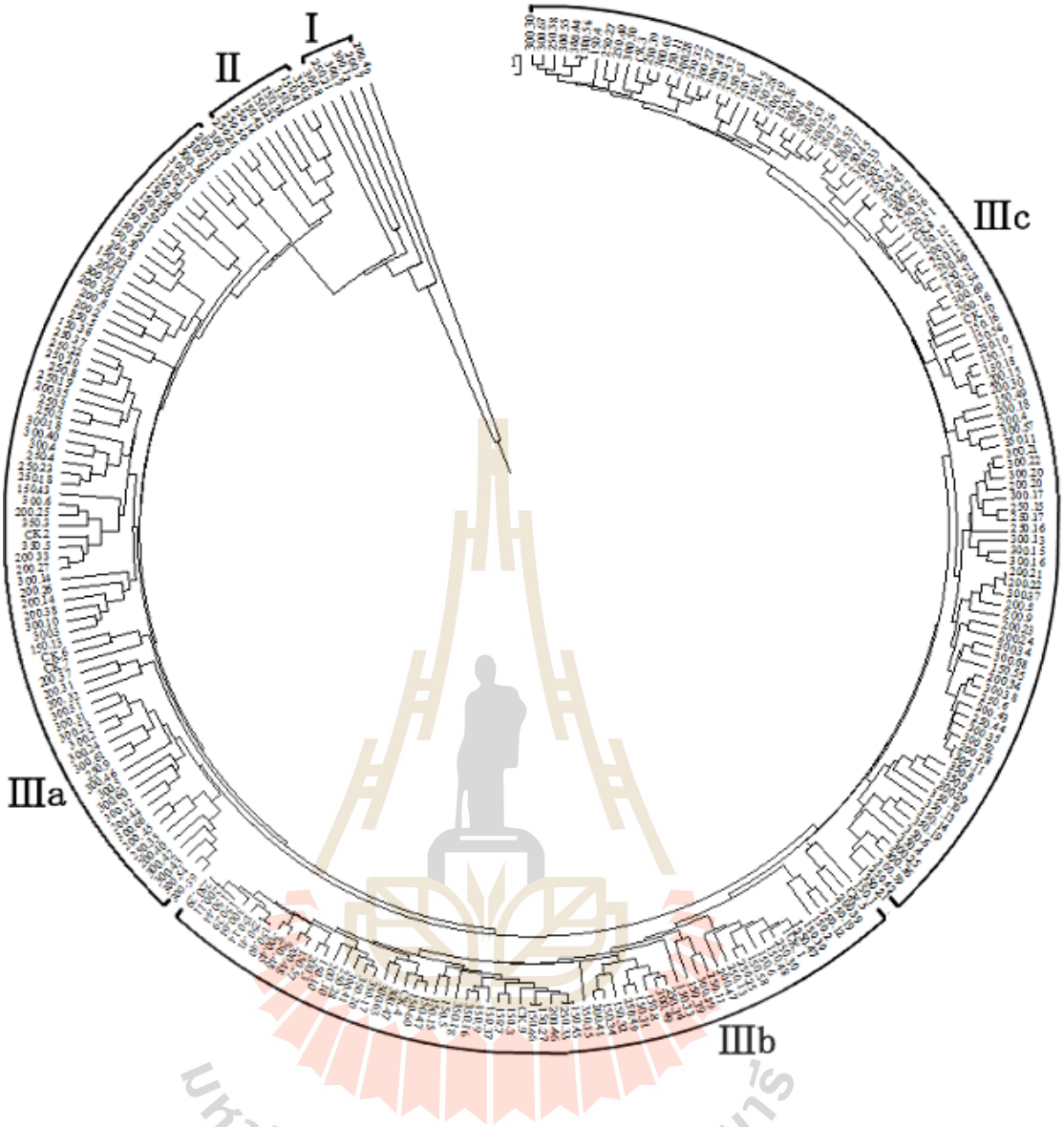

Figure 5.6 UPGMA dendrogram generated from ISSR data showing relationships of 265 samples of Y159 which were irradiated by ^{60}Co-γ radiation.

Note: 150 to 350 represents 150 to 350Gy, CK represents control (0 Gy).

As a result, a total of 57 DNA fragments were amplified from the 204 EMS-treated samples using the five primers, and 37 of them were polymorphic (64.9%). Five to nine polymorphic DNA bands were amplified with each primer, with an average of 7.4 polymorphic DNA bands per primer. Nine polymorphic bands were produced with

either UBC836 or UBC857, and the percentages of polymorphism were 81.8% and 64.3%, respectively. Only five polymorphic bands were generated with UBC856, and the percentage of polymorphism was 50.0% (Table 5.6, Figure 5.7 and Figure 5.8).

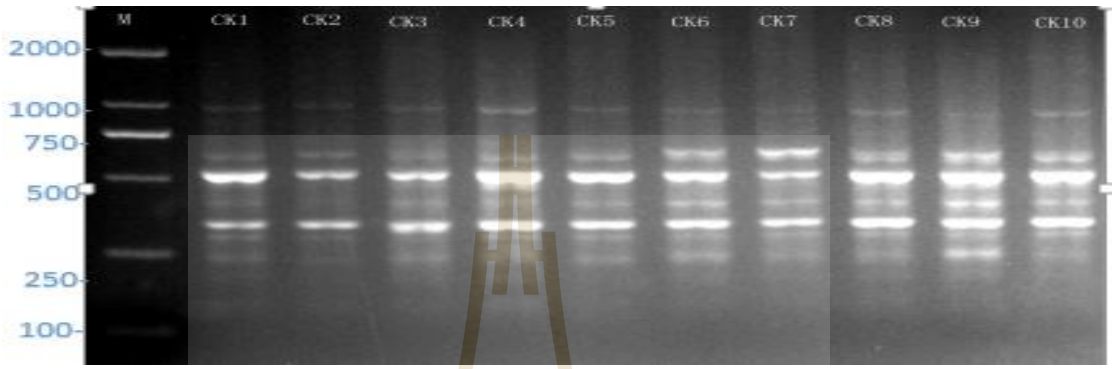

Figure 5.7 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836 .

Note: CK1 to CK10 represents control samples number.

Figure 5.8 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in garose gel using the primers UBC83.

Note: A1 to A22 represents 0.4% EMS concentration treated 1 to 22 samples number, B1 to B2 represents 0.8% EMS concentration treated 1 to 2 samples number.

Table 5.6 Inter simple sequence repeat primers and amplification results by using EMS solution.

Primer	Sequence (3' –5')	Optimal annealing temperature	Number of total amplified bands	Number of polymorphic bands (NPB)	Percentage of polymorphic Loci (PPB)
UBC818	CAC ACA CAC ACA CAC AG	56.0	13	8	61.5%
UBC836	AGA GAG AGA GAG AGA GYA	53.9	11	9	81.8%
UBC841	GAG AGA GAG AGA GAG AYC	54.7	9	6	66.7%
UBC856	ACA CAC ACA CAC ACA CYA	54.7	10	5	50.0%
UBC857	ACA CAC ACA CAC ACA CYG	56.2	14	9	64.3%
Total			57	37	64.9%
Average		55.1	11.4	7.4	

Y=(C, T), R= (A, G)

5.4.6.2 Cluster analysis

Cluster analysis showed that 204 samples treated with different concentrations of EMS were divided into three groups. Group I composed of 20 samples, including one sample treated with each of 0.4, 0.8, 1.2, 3.6, 4.0, 4.4, 4.8, and 5.2% EMS concentrations, three samples treated with 2.4% EMS, six samples treated with 2.8% EMS, three samples treated with 3.2% EMS, and almost all the samples treated with EMS solutions at 4.0% and above (Figure 5.9-I). Group II consisted of 43 samples, including one sample treated with 0.8% EMS (A4), one sample of the control (CK4), 20 samples treated with 0.8% EMS, and 21 samples treated 1.2% EMS. The 20 samples treated with 0.8% EMS accounted for 76.9% of all the samples in this treatment (20/26), and the 21 samples treated with 1.2% EMS accounted for 87.5% of all the samples in this treatment (21/24) (Figure 5.9-II). Group III consisted of 141 samples, including all the control samples except for CK4. Group III could be further divided into two subgroups. Subgroup IIIa was composed of 60 samples, including 17 samples treated with 2.8% EMS, and 24 samples treated with 3.2% EMS (Figure 5.9-IIIa). The samples treated with the two concentrations of EMS accounted for 68.3% of all the samples in Subgroup IIIa, and the samples of each concentration clustered together. Subgroup IIIab was composed of 81 samples, including 81.8% of the samples treated with 0.4% EMS (18/22), all the samples treated with 1.6% EMS (14/14), and 80% of the samples treated with 2.0% EMS (24/30).

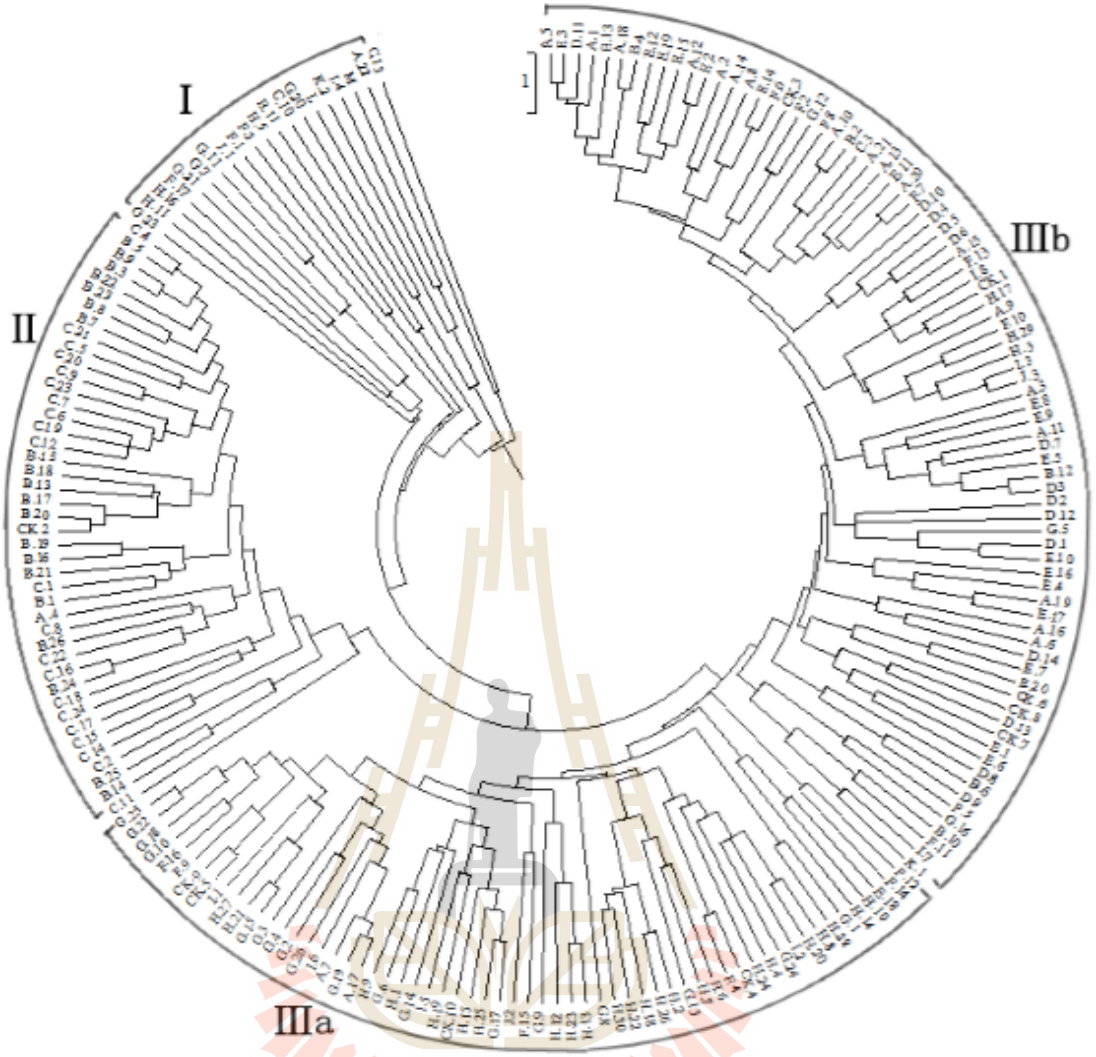

Figure 5.9 UPGMA dendrogram generated from ISSR data showing relationships of 204 samples of Y159 which were irradiated by EMS solution.

Note: A to E represents 0.4% to 5.2% EMS concentrations, CK represents control (0 %).

5.5 Discussion

Induced mutagenesis is a tool to create new variability in any crop with a higher frequency than the spontaneous mutations (Chopra, 2005). After the mutagenic treatments, an inhibitory effect on seed germination could be distinctly seen in Job's tears. Germination of Job's tears decreased with the increase in concentration of

mutagenic treatments. The reduction percentage in seed germination had a gradual decreasing trend from lower to higher doses，this results similar to Datir et al. (2007) in horsegram and Potdukhe and Narkhede (2002) in pigeon pea. The reduction percentage in seed germination might have been due to the effect of mutagens on meristematic tissues of the seed (Ariraman, et al, 2014).

The decrease in seed germination at higher concentration of the mutagens maybe attributed to disturbances at cellular level (caused either at physiological (or) physical level). Kumar and Mishra (2004) reported that in okra (*Abelmoschus esculentus*) germination percentage generally decreased with increasing concentrations of gamma rays and EMS. Reduced germination percentage with increasing doses of gamma radiation has also been reported in Pinus (Thapa, 2004), Rye (Akgun and Tosum, 2004) and Chickpea (Khan et al., 2005; Toker et al., 2005).

In this study, there was a phenomenon which lower doses promotes seed germination and high doses inhibits germination, and the time required for germination to complete also increases with the increase of the dose. Therefore, select the appropriate dose of mutagenesis is the key to success of mutation breeding. For artificially induced mutations either with physical or chemical mutagens, LD 50 has been considered as the best dose for high frequency of mutations (Anbarasan et al., 2013). In the present investigation, the LD50 was calculated on the basis of seed germination at different doses of Gamma rays and EMS (Yadav et al., 2016). In the present investigation, the LD50 of two Job's tears varieties CDT and Y159 was found at 406.305Gy and 284.795 Gy of gamma ray dosages, respectively. The LD50 of Y159 Job's tears varieties was found at 2.453% of gamma ray EMS dosages.

Research results show that the effects of different treatment doses on the growth and variation of seedlings were significant, mainly reflected in the variation of seedling height and leaf type. Gamma rays and EMS was drastically reduced the seedling height in Job's tears at higher concentrations. Similar observations were made in sunflower (Jayakumar and Selvaraj 2003). The stimulatory effect was observed in lower doses of gamma rays and EMS on the seedling height, these resuts maybe due to the cell division rates as well as an activation of growth hormone, e.g., auxin (Zaka et al., 2004).

In this study, the plants derived from radiation and EMS mutagenesis were also analyzed at the molecular level using the ISSR markers. Among various molecular markers, ISSR is based on PCR amplification of DNA (Reddy et al., 2002; Semagn et al., 2006). ISSR markers were used to detect the differences among mutants in different species. For example, this technique was successfully used to show genetic differences between control and putative banana mutants (Khatri et al., 2011; Semagn et al., 2006). ISSR markers were used to show polymorphism ratios and genetic differences between gamma radiation-induced mutants in soybean (Mudibu et al., 2011). For the ISSR analysis with 5 primers for the irradiated Y159 samples of Job's tears a total of 57 polymorphic bands were obtained whereas 34 (EMS treated was 37) were monomorphic with an average of 11.4 total bands and 6.8 (EMS treated was 7.4) polymorphic bands, respectively. The largest number of bands was identified in primer UBC857 (14 bands) and the smallest in primers UBC857 (9 bands). The Percentage of polymorphic bands were 59.6% in Gamma rays and 64.9% in EMS. This was indicated that the Job's tears has large variation and rich genetic diversity after mutagenesis treated, and EMS have more effective and efficient than Gamma rays mutagens (John et al., 1999).

Cluster analysis revealed that 265 gamma-irradiated Y159 samples were divided into three groups (Figure. 5.21). All the control samples were clustered into Group III which showed that there was a small genetic distance between the gamma-irradiated samples in this group and the control. The result suggested that little genetic variation was generated in these gamma-irradiated samples. No control sample was included in Group II and Group III, indicated that a larger genetic distance between the gamma-irradiated samples in the two groups and the control, which further proved that there was significant genetic variation in theses samples. The genetic distance between Group I samples and the control was larger than that between Group II samples and the control, suggested that Group I had a higher degree of genetic variation than Group II. 15 samples in Group I and Group II accounted for only 5.7% of all samples, indicated that gamma radiation generated a low mutation rate in Y159. A larger genetic distance indicates a higher degree of genetic variation. There were 150 to 350 Gy-treated samples in Group I and Group II, and no 350 Gy- treated samples in Group I, which showed the highest degree of genetic variation, indicated that all these doses of gamma radiation could generate mutations in Job's tears, and the variation level was not totally determined by radiation dose.

Cluster analysis showed that the 204 samples treated with different concentrations of EMS were divided into three groups (Figure. 32). Group III samples had the smallest genetic distance from the control samples, followed by Group II samples, and Group I samples had the largest genetic distance from the control samples. A larger genetic distance indicates a higher degree of genetic variation. Hence, the samples in Group I and Group II had higher degree of genetic variation. In Group I, the samples M, L, K2, and I4 were treated with high concentrations of EMS (more than 4.0%), the samples A22, B11, and C1 were treated with low concentrations

of EMS (less than 1.2%), and the samples F1, F3. F17, G7, G11, G20, G21, G23, H5, H11 and H16 were treated with medium concentrations of EMS. The results also showed that EMS at all the concentrations were able to generate mutations in Job's tears, and the degree of genetic variation was not totally determined by EMS concentration. A high degree of genetic variation could be produced using a low concentration of EMS, but there was a larger possibility to obtain a high degree of genetic variation when the seeds were treated with EMS at a higher concentration.

5.6 Conclusion

Low-dose ^{60}Co-γ radiation was able to increase seed germination rate and the effect of radiation on germination rate varied among the Job's tears cultivars. The dose of ^{60}Co-γ radiation and the concentrations of EMS had a significant impact on seed germination rate, seedling heights and mutation rate. The seed germination rate and seedling heights gradually decreased with increasing the dose of ^{60}Co-γ radiation and the concentrations of EMS. The Mutation rate of Job's tears gradually increased with increasing the dose of ^{60}Co-γ radiation and the concentrations of EMS.

The regression equations for ^{60}Co-γ radiation irradiated CDT and Y159 were: Probit=2.222-0.005X and Probit=2.053-0.007X, and their LD50 were 406.305 and 284.795 Gy, respectively. The regression equation for EMS-treated Y159 was Probit=1.807-0.737X, and the LD50 was 2.453%.

A total of 57 DNA fragments were amplified from the 265 samples ^{60}Co-γ radiation irradiated and 204 samples EMS treated by using the five primers. 34 of the DNA fragments were polymorphic (59.6%) with an average of 6.8 polymorphic DNA bands per primer in ^{60}Co-γ radiation experiment. 34 of the DNA fragments were

polymorphic (64.9%) with an average of 7.4 polymorphic DNA bands per primer in EMS experiment.

Cluster analysis revealed that the 265 ^{60}Co-γ radiation irradiated samples were divided into three groups, the samples in Group I had a higher degree of genetic variation than Group II and Group III. Cluster analysis showed that the 204 samples treated with different concentrations of EMS were divided into three groups. The samples in Group I and Group II had higher genetic variation than Group III. Both high and low mutagens were able to generate mutations in Job's tears. However, the degree of genetic variation was not totally determined by EMS concentration. A high of genetic variation could be produced using a lower dose of 60Co-γ radiation and lower concentration of EMS, but there was a larger possibility to obtain a high genetic variation when the seeds were treated with mutagen of ^{60}Co-γ radiation and EMS at a higher level.

5.7 References

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CHAPTER VI

CONCLUTION

The genetic diversity assessment and study on induced mutation of Job's tears were studied in three experiments, and the results can conclused as follows:

Experiment I (Genetic diversity analysis based on morphological traits)

1. Morphological diversity studies on Job's tears were carried out in 94 accessions. The results showed high variation among the studied materials from ANOVA, simple correlations and multivariate analysis. Significant and positive correlation were found between grain number per plant and among other yield-related attributes, it indicated that these accessions have the potential for improvement yield of Job's tears.

2. The principal component analysis showed that the variations observed in the accessions are mainly caused by traits such as total bract surface characteristics，total bract texture, total bract shape, plant height, stem node number and primer branch nodes, indicating that their contribution was important in discriminating the accessions.

3. Cluster analysis grouped 94 accessions of Job's tears into seven clusters, the results showed that the accessions collected from same or different place can be grouped together, and the different type of variety (wild or cultivated) or different qualitative traits can be also grouped together, it indicated high diversity for most of

the traits, demonstrating that genetic differences can not only based on the geographical differences, type of variety (wild or cultivated) and qualitative traits.

Experiment II (Genetic diversity analysis based on ISSR marker)

1. 10 pairs of ISSR primers screened in this study, had 98 of the DNA fragments were polymorphic (84.48%) with an average of 9.8 polymorphic bands per primer, indicating that the selected primers have higher polymorphism and suitable for genetic diversity analysis for Job's tears.

2. In the analysis of genetic diversity and genetic structure of eight populations of Job's tears, the results showed that populations of Job's tears have more abundant genetic diversity. The Guizhou population has the highest genetic diversity, while the Hubei population has the lowest genetic diversity.

3. Both genetic distance and UPGMA cluster analysis among the eight populations of Job's tears shows that populations between Guizhou and Chongqing, Guangxi and Sichuan, Yunnan and Qianxinan Institute have close genetic relationship. Both results between Gst and AMOVA showed that major proportion of the total variation of job's tears existed within populations and the minor variations existed among populations.

4. Both Bayesian cluster and UPGMA cluster analysis among individuals of Job's tears have largely consistent results. 94 accessions of Job's tears were grouped into two Group. The results showed that accessions of Job's tears clustered normally according collect areas except a few accessions.

5. The results of mantel test showed that there was no correlation between geographic distance and genetic distance among the populations ($r = 0.055$, $p = 0.782$), genetic drift plays a greater role in population structure than gene flow.

Experiment III (Induced mutation)

1. ^{60}Co-γ radiation doses and the concentrations of EMS had a significant impact on seed germination rate, seedling heights and Mutation rate. The seed germination rate and seedling heights gradually decreased with the increasing dose of 60Co-γ radiation and the concentrations of EMS. The Mutation rate of Job's tears gradually increased with increasing the dose of 60Co-γ radiation and the concentrations of EMS.

2. The regression equations for gamma-irradiated CDT and Y159 were: Probit=2.222-0.005X and Probit=2.053-0.007X, and their LD50 were 406.305 and 284.795 Gy, respectively. The regression equation for EMS-treated Y159 was Probit=1.807-0.737X, and the LD50 was 2.453%.

3. Analysis of genetic diversity results showed that samples of Job's tears which were treated by ^{60}Co-γ radiation and EMS have rich genetic diversity. The percentage of polymorphic were 59.6% and 64.9%, respectively.

4. Cluster analysis revealed that both high or low mutagens were able to generate mutations in Job's tears. A high of genetic variation could be produced by using a lower dose of ^{60}Co-γ radiation and lower concentration of EMS, but there was a larger possibility to obtain a high genetic variation when the seeds were treated with mutagen of ^{60}Co-γ radiation and EMS at a higher level.

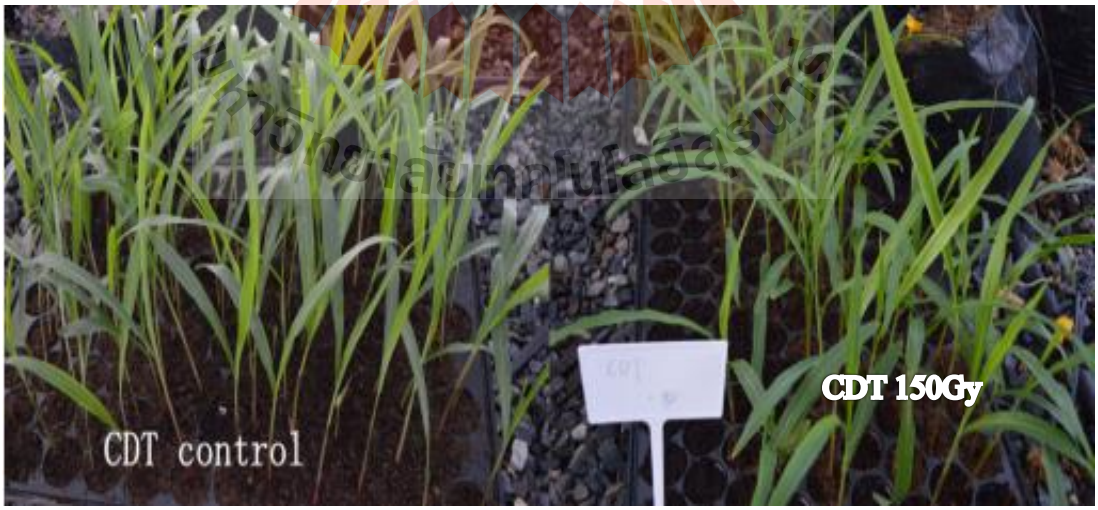
In conclusion, both morphological and molecular diversity indicated that there has abundant genetic diversity in Job's tears germplasm resources. Although the clustering results of 94 accessions which were based on both morphological marker and ISSR marker were different, both the results showed that the accessions collected from same or different place can be grouped together, and genetic differences can not

only based on the geographical differences. Due to morphological traits are easily affected by environmental conditions, the genetic diversity assessment need combine both morphological and molecular methods. Mutagenesis experiments revealed the LD50 of both CDT and Y159 cultivars. The results showed that samples of Job's tears which were treated by ^{60}Co -γ radiation and EMS have rich genetic diversity. Therefore, this study can provide reference for the breeding of Job's tears.

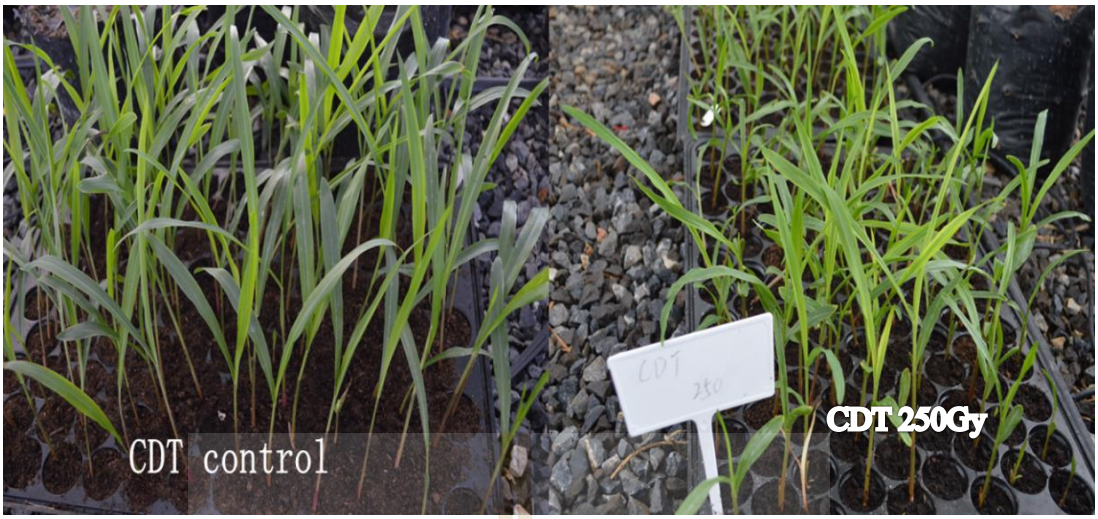
APPENDIX

I. ATTACHED FIGURES

Attached figure 1 The germplasm resource garden of Job's tears.



Attached figure 2 The control and 150 Gy of ^{60}Co-γ- irradiated CDT variety.

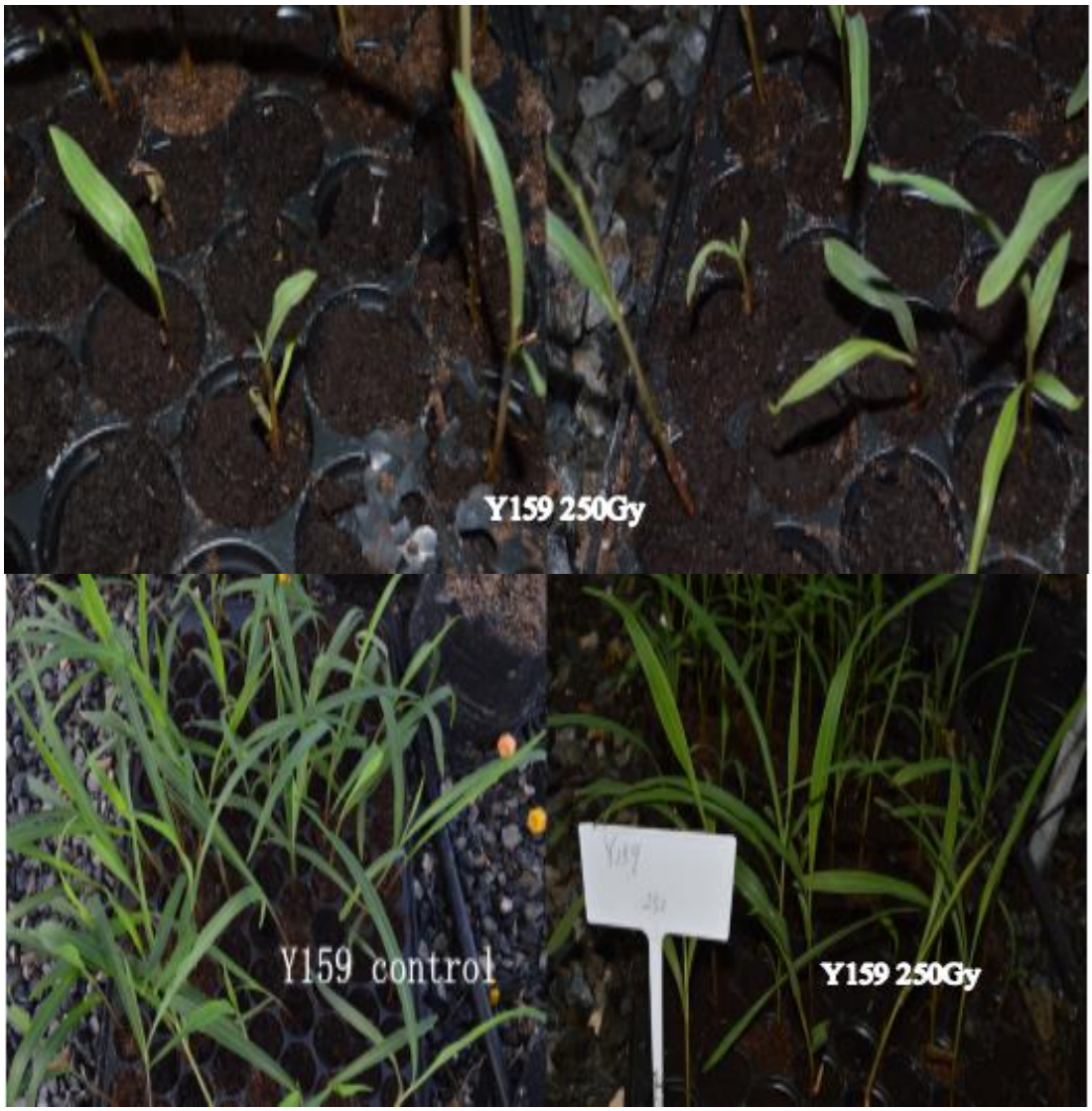


Attached figure 3 The control and 250 Gy of ^{60}Co-γ- irradiated CDT variety**.**

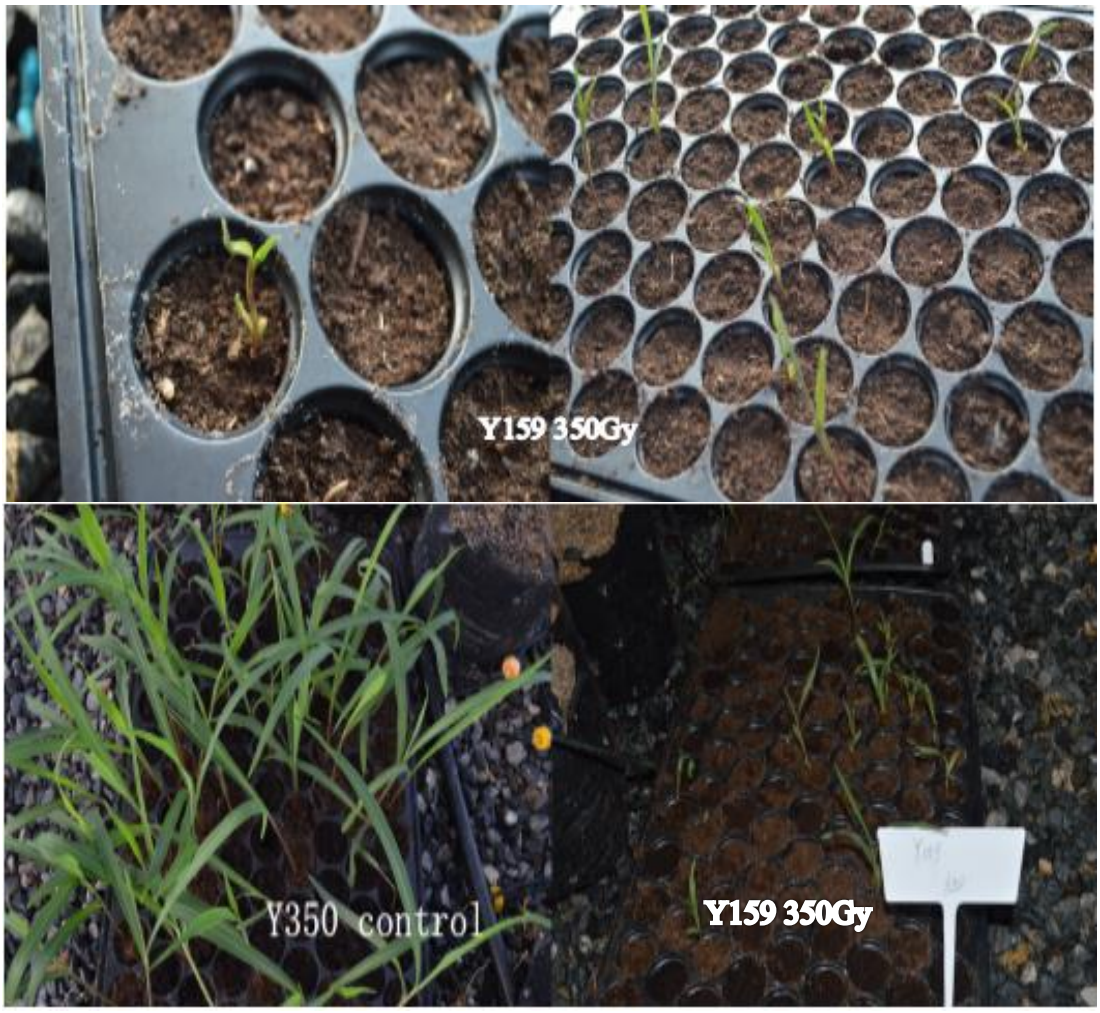
Attached figure 4 The control and 350 Gy of ^{60}Co-γ- irradiated CDT variety.

Attached figure 5 The control and 450 Gy of ^{60}Co-γ- irradiated CDT variety.

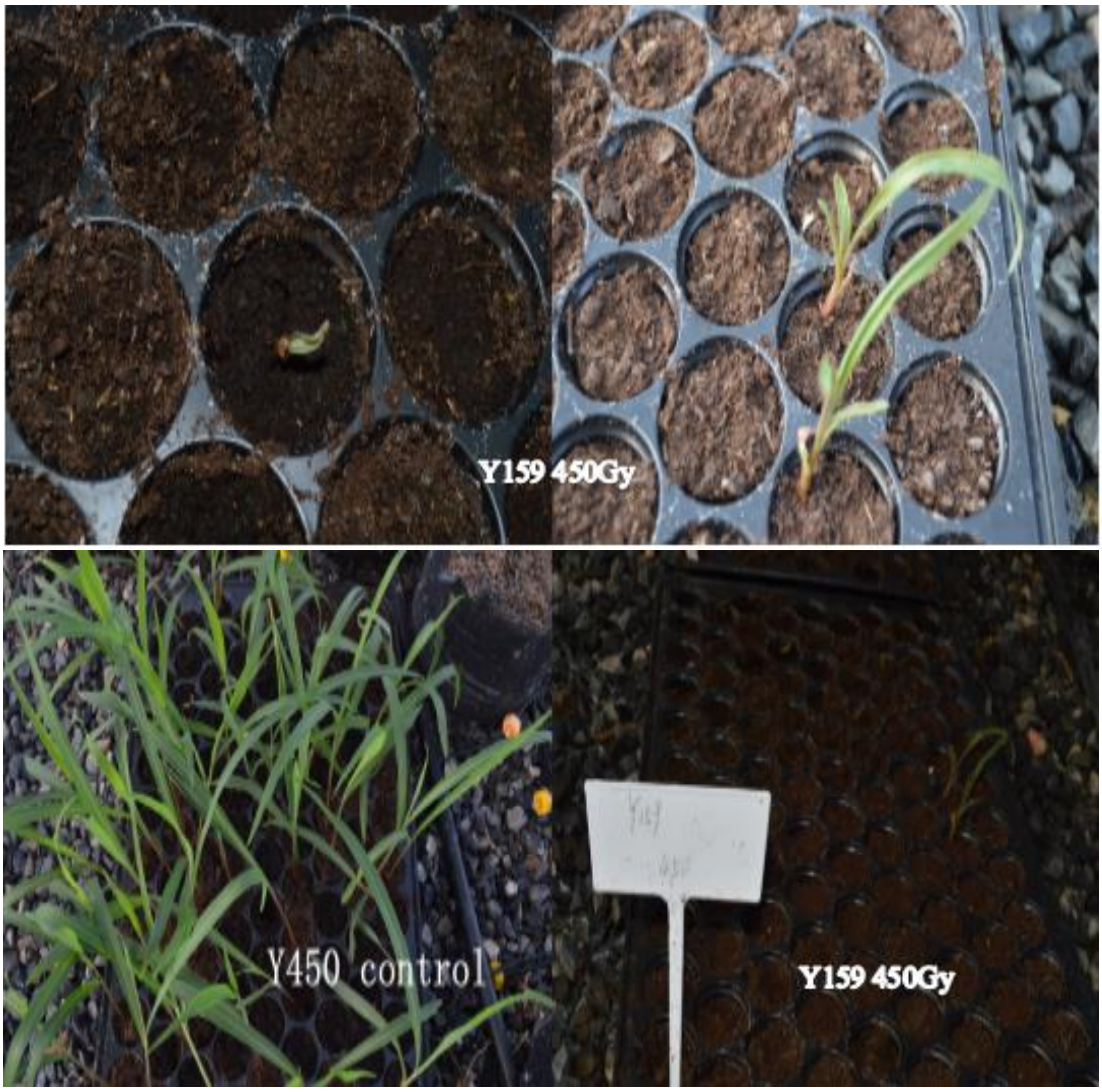
Attached figure 6 The control and 150 Gy of ^{60}Co-γ- irradiated Y159 variety.



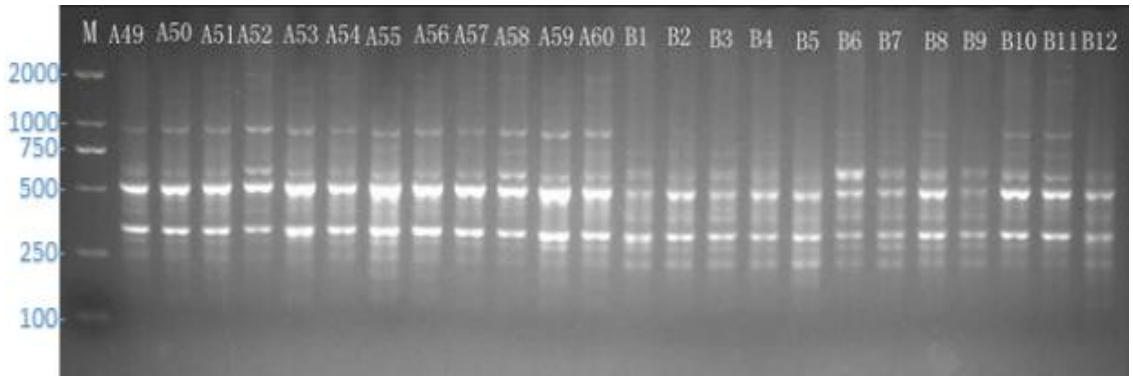
Attached figure 7 The control and 250 Gy of ^{60}Co-γ- irradiated Y159 variety.



Attached figure 8 The control and 350 Gy of ^{60}Co-γ- irradiated Y159 variety.

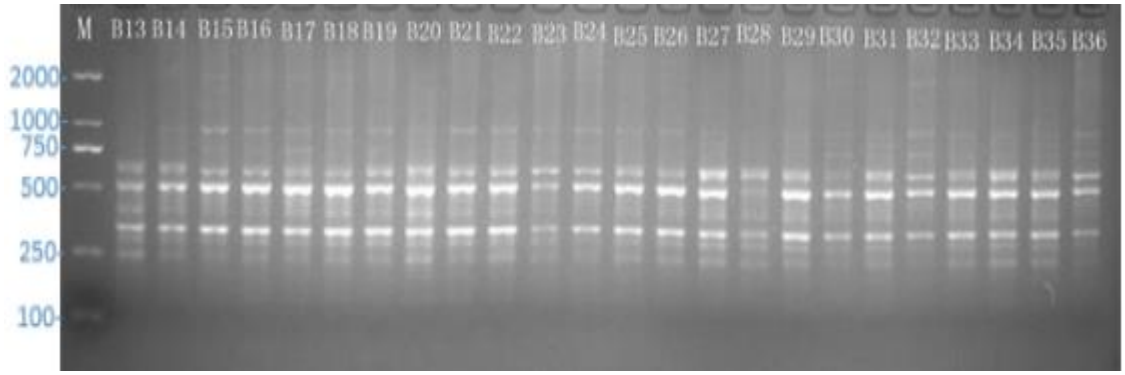


Attached figure 9 The control and 450 Gy of ^{60}Co-γ- irradiated Y159 variety.



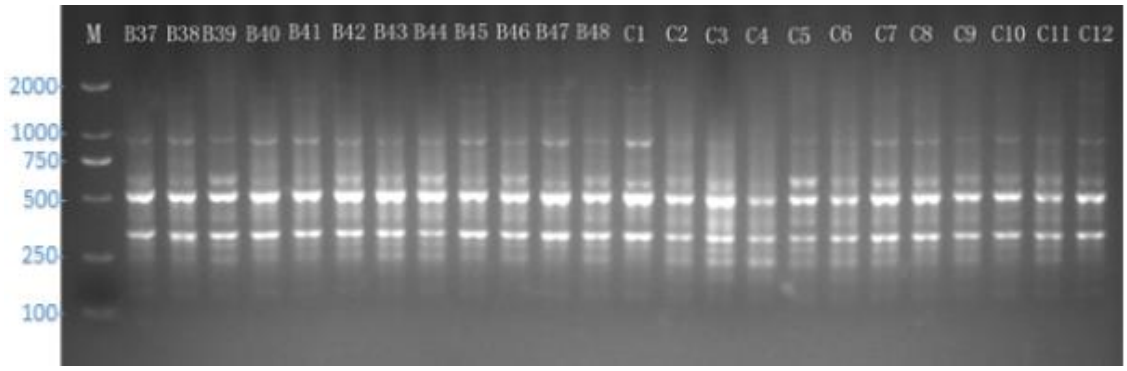
Attached figure 10 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: A49 to A60 represents 150 Gy irradiated 25 to 48 samples number, B1 to B12 represents 200 Gy irradiated 1 to 12 samples number.



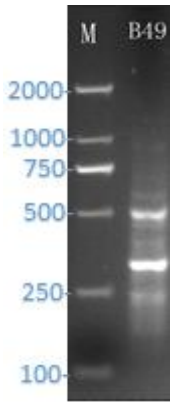
Attached figure 11 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: B13 to B36 represents 200 Gy irradiated 13 to 36 samples number.



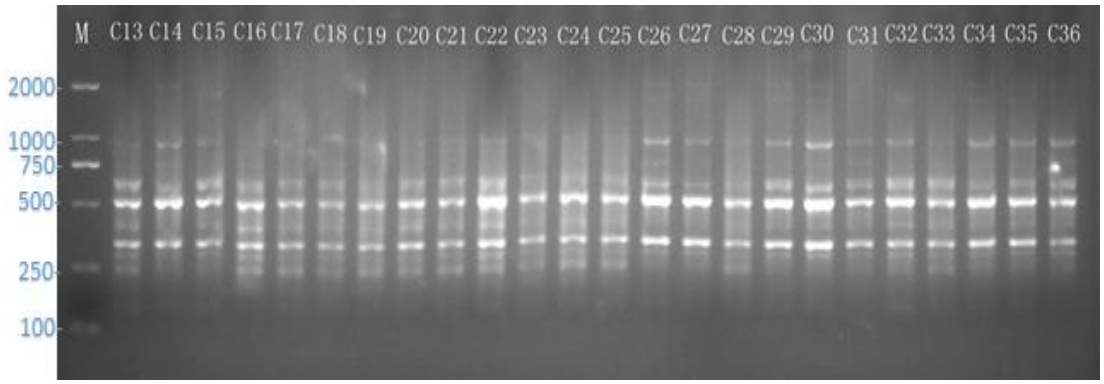
Attached figure 12 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: B37 to B48 represents 200 Gy irradiated 37 to 48 samples number, C1 to C12represents 250 Gy irradiated 1 to 12 samples number.



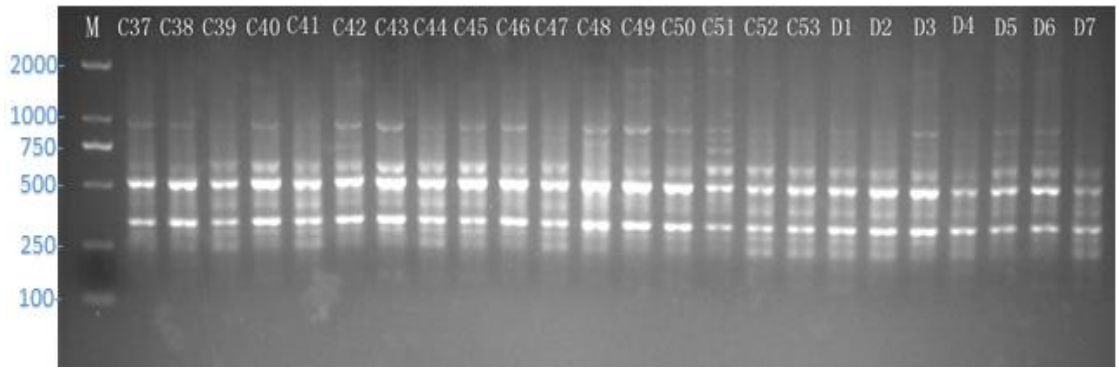
Attached figure 13 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: B49 represents200 Gy irradiated sample number



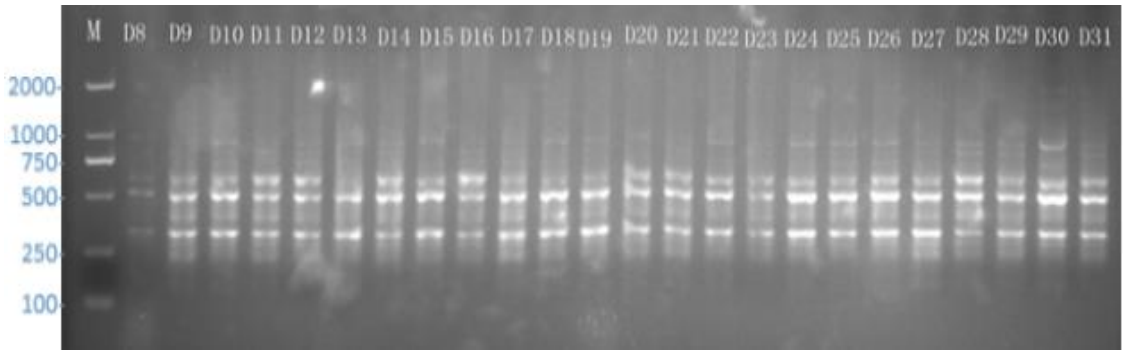
Attached figure 14 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: C13 to C36 represents 250 Gy irradiated 13 to 36 samples number.



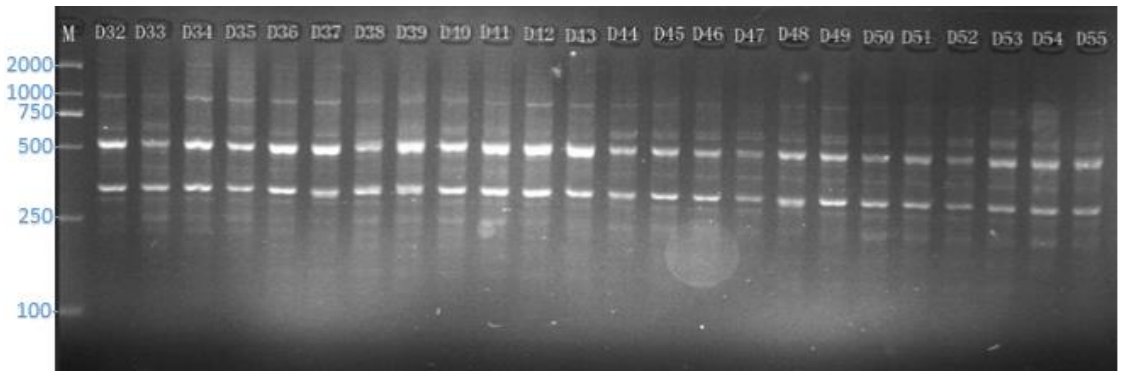
Attached figure 15 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: C37 to C53 represents 250 Gy irradiated 37 to 53 samples number, D1 to D7 represents 300 Gy irradiated 1 to 7 samples number.



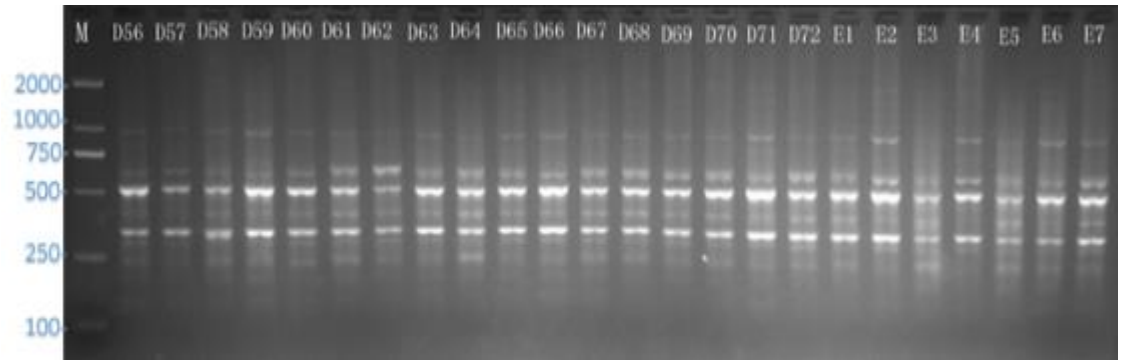
Attached figure 16 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: D8 to D31 represents 300 Gy irradiated 8 to 31 samples number.



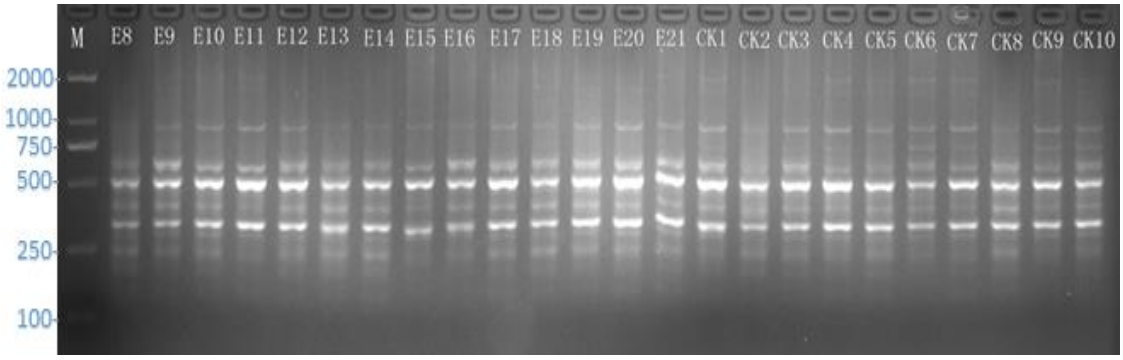
Attached figure 17 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: D32 to D55 represents 300 Gy irradiated 32 to 55 samples number.



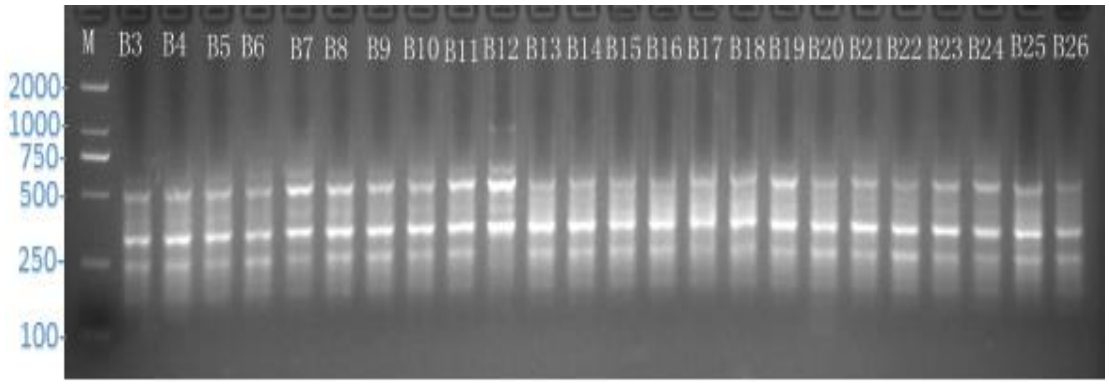
Attached figure 18 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: D56 to D72 represents 200 Gy irradiated 56 to72 samples number, E1 to E7 represents 350 Gy irradiated 1 to 7 samples number.



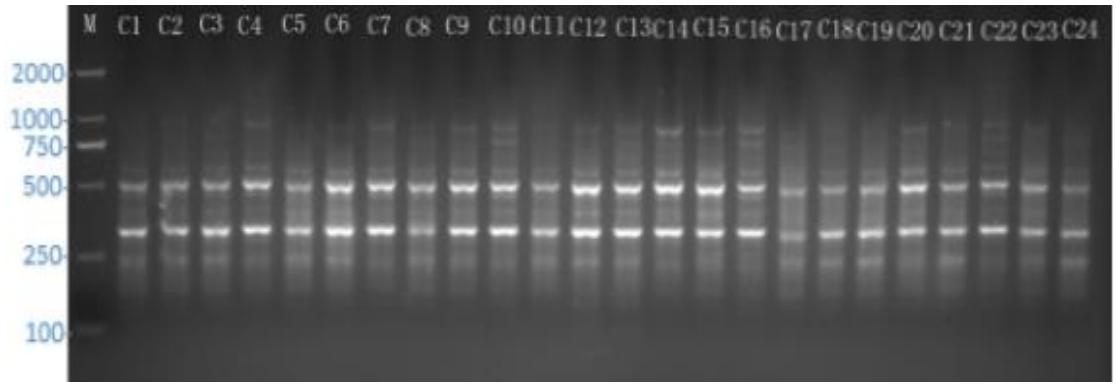
Attached figure 19 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: E8 to E21 represents 350 Gy irradiated 8 to 21 samples number, CK1 to CK10 repre sents control samples number.



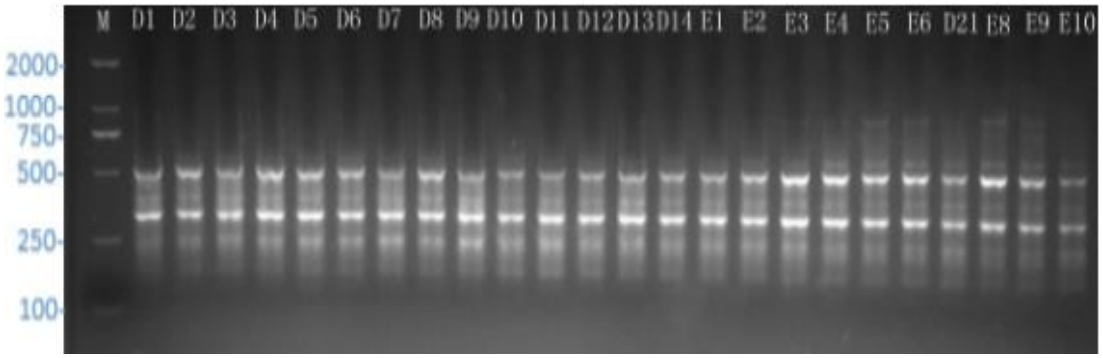
Attached figure 20 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: B3 to B26 represents 0.8% EMS concentration treated 3 to 26 samples number.



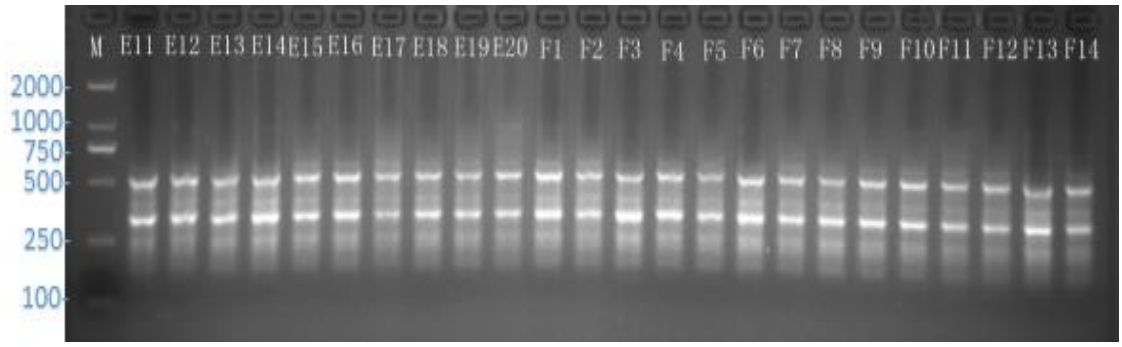
Attached figure 21 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: C1 to C24 represents 1.2% EMS concentration treated 1 to 24 samples number.



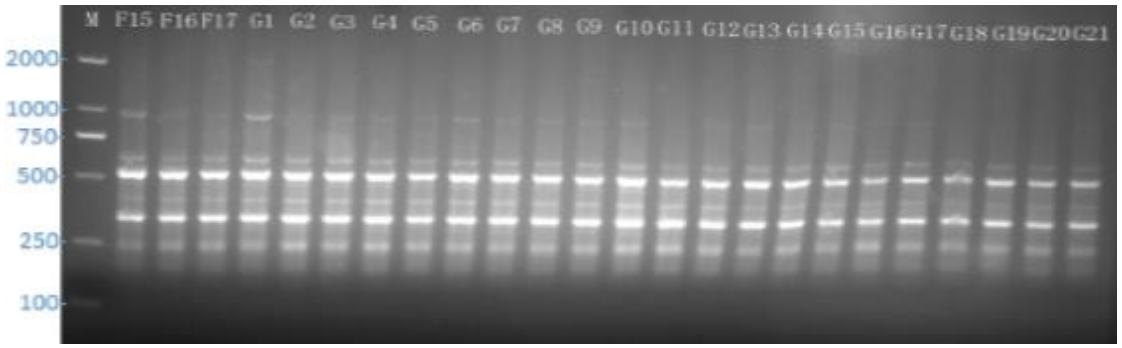
Attached figure 22 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: D1 to D14 represents 1.6% EMS concentration treated 1 to 14 samples number, E1 to E10 represents 2.0% EMS concentration treated 1 to 10 samples number.



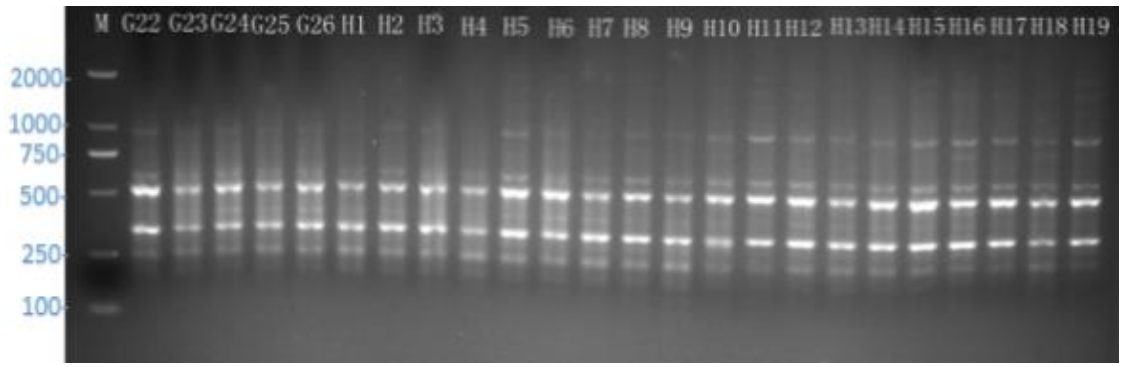
Attached figure 23 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: E11 to E20 represents 2.0% EMS concentration treated 11 to 20 samples number, F1 to F14 represents 2.4% EMS concentration treated 1 to 14 samples number.



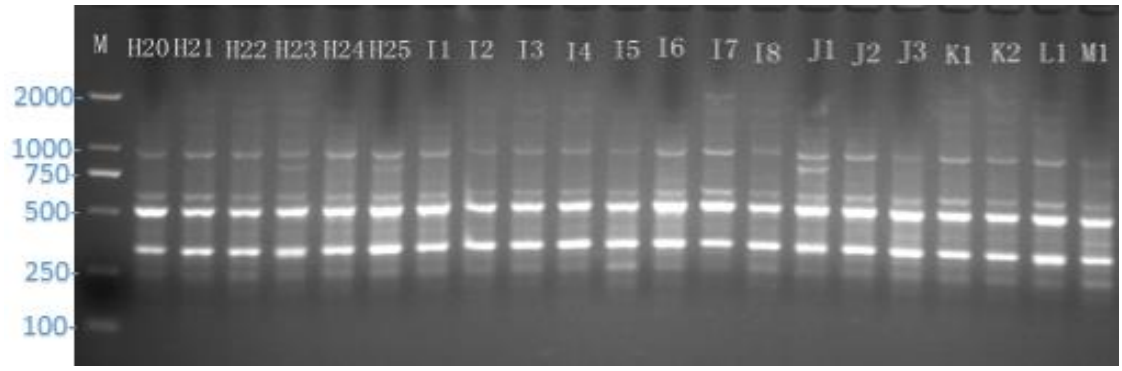
Attached figure 24 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: F15 to F18 represents 2.4% EMS concentration treated 15 to 18 samples number, G1 to G21 represents 2.8% EMS concentration treated 1 to 21 samples number.



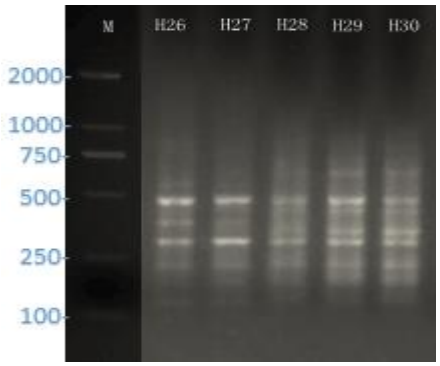
Attached figure 25 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: G22 to G26 represents 2.8% EMS concentration treated 22 to 26 samples number, H1 to H19 represents 3.2% EMS concentration treated 1 to 19 samples number.



Attached figure 26 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: H20 to H25 represents 3.2% EMS concentration treated 20 to 25 samples number, I1 to I8 represents 3.6% EMS concentration treated 1 to 8 samples number, J1 to J3 represents 4.0% EMS concentration treated 1 to 3 samples number, K1 to K2 represents 4.4% EMS concentration treated 1 to 2 samples number, L1 and M1 represents 4.4% and 5.2% EMS concentration treated samples number.



Attached figure 27 Electrophoretic profiles of ISSR of Job's tears genotypes amplified in agarose gel using the primers UBC836.

Note: H26 to H30 represents 3.2% EMS concentration treated 26 to 30 samples number.

Attached figure 28 Plant morphology of Job's tears.

II. ATTACHED TABLES

Attached table 1 The conversion value of 5 qualitative morphological traits.

Code	TBSC	TBT	TBS	TBC	PC	Code	TBSC	TBT	TBS	TBC	PC
Y1	1	1	2	6	1	Y48	1	1	2	6	2
Y2	1	1	2	8	3	Y49	1	1	2	6	1
Y3	1	1	2	8	3	Y50	1	1	2	8	2
Y4	1	1	2	2	3	Y51	1	1	2	8	3
Y5	1	1	2	7	1	Y52	2	2	3	4	1
Y6	2	2	3	1	1	Y53	1	1	2	2	3
Y7	1	1	2	9	1	Y54	1	1	2	9	1
Y8	1	1	2	4	1	Y55	2	2	3	1	1
Y9	1	1	2	8	2	Y56	1	1	2	9	3
Y10	1	1	1	5	3	Y57	2	2	3	1	1
Y11	1	1	2	8	3	Y58	2	2	3	9	3
Y12	1	1	2	7	1	Y59	2	2	3	1	1
Y13	1	1	3	7	1	Y60	2	2	3	1	1
Y14	1	1	2	9	3	Y61	2	2	3	9	3
Y15	1	1	2	9	3	Y62	2	2	1	3	1
Y16	1	1	2	8	2	Y63	2	2	3	9	1
Y17	2	2	3	1	1	Y64	2	2	3	1	1
Y18	1	1	2	8	2	Y65	2	2	3	1	1
Y19	1	1	2	9	3	Y66	2	2	3	1	1
Y20	1	1	2	4	1	Y67	2	2	2	1	1
Y21	1	1	2	7	1	Y68	2	2	2	1	1
Y22	1	1	2	2	2	Y69	1	1	1	9	3
Y23	1	1	2	2	2	Y70	1	1	2	9	3
Y24	1	1	2	9	3	Y71	1	1	2	9	3
Y25	2	2	3	1	1	Y72	1	1	2	9	3

TBSC: Total bract surface characteristics TBT: Total bract texture TB: Total bract shape TBC: Total bract color PC: Pericarp color.

Attached table 1 The conversion value of 5 qualitative morphological traits (Continued).

Code	TBSC	TBT	TBS	TBC	PC	Code	TBSC	TBT	TBS	TBC	PC
Y26	2	2	3	1	1	Y73	1	1	2	8	1
Y27	1	1	2	8	3	Y74	1	1	2	9	3
Y28	1	1	2	8	2	Y75	1	1	2	9	3
Y29	2	2	3	8	1	Y76	2	2	1	9	1
Y30	2	2	3	1	1	Y77	2	2	3	8	3
Y31	1	1	2	7	1	Y78	2	2	3	8	3
Y32	1	1	2	7	1	Y79	1	1	2	2	3
Y33	1	1	2	3	3	Y80	2	2	2	1	1
Y34	1	1	2	9	3	Y81	2	2	2	6	1
Y35	1	1	2	2	3	Y82	2	2	3	8	3
Y36	2	2	3	1	1	Y83	2	2	3	1	1
Y37	2	2	3	1	1	Y84	1	1	2	9	3
Y38	2	2	3	1	1	Y85	1	1	2	8	1
Y39	2	2	3	1	1	Y86	2	2	3	8	1
Y40	2	2	3	1	1	Y87	1	1	1	9	1
Y41	2	2	3	9	3	Y88	2	2	3	9	3
Y42	2	2	3	1	3	Y89	2	2	3	1	1
Y43	2	2	3	1	1	Y90	2	2	3	1	2
Y44	1	1	2	8	2	Y91	2	2	3	1	1
Y45	1	1	2	8	2	Y92	1	1	2	1	3
Y46	1	1	2	3	1	Y93	1	1	2	9	1
Y47	1	1	2	9	2	Y94	1	1	2	9	3

TBSC: Total bract surface characteristics TBT: Total bract texture TB: Total bract shape TBC: Total bract color PC: Pericarp color.

BIOGRAPHY

Mr. Gang Shen was born on October 01, 1981 in Wuchuan county, Guizhou province, P. R. China. He holds a BA degree in Biological Science and a M.S degree in Botany from Guizhou Normal University. Mr. Gang Shen is currently a Ph. D. candidate in plant breeding under the supervision of Dr. Teerayoot Girdthai in the School of Crop Production Technology, Institute of Agriculture Technology, Suranaree University of Technology, Thailand.