A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Genetic diversity of Indian lettuce (*Lactuca indica* Linn.) and *in vitro* propagation

BY

Bal Kumari Oliya

August, 2018

MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY

DEPARTMENT OF PLANT SCIENCE

THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSIT

Genetic diversity of Indian lettuce (*Lactuca indica* Linn.) and *in vitro* propagation

UNDER THE DIRECTION OF DR. SUK-HA LEE
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

BY

Bal Kumari Oliya

MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY
DEPARTMENT OF PLANT SCIENCE

MAY, 2018

APPROVED AS A QUALIFIED DISSERTATION OF
BAL KUMARI OLIYA
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
BY THE COMMITTEE MEMBERS

JULY, 2018

CHAIRMAN	Byeon Woo Lee, Ph.D.
VICE-CHAIRMAN	Suk-Ha Lee, Ph.D.
MEMBER	Hak Soo Seo, Ph.D.
MEMBER	Do-Soon Kim, Ph.D.
MEMBER	Jae Gyun Gwag, Ph.D.

To

My beloved parents, lovely husband and beautiful daughters

Genetic diversity of Indian lettuce (*Lactuca indica* Linn.) and *in vitro* propagation

Bal Kumari Oliya

DEPARTMENT OF PLANT SCIENCE

THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

General abstract

Indian lettuce (*Lactuca indica* L.) is an important undomesticated medicinal plant in the Asteraceae family. Recently, transcriptomic and metabolic data for this plant have made available which provided a major boost for genomic, breeding and pharmacological research. It is important to investigate genetic diversity to this wild plant for breeding. In addition to this, development of *in vitro* propagation protocol could help for commercial cultivation and various biotechnological breakthrough. Therefore, this study first developed genic-simple sequence repeat (SSR) markers using transcriptome sequence data, to study the

genetic diversity and marker transferability then, investigated the morphological characteristics for analyzing genetic variability and agronomic performance. The newly developed genic-SSRs were highly polymorphic and yielded an average of 10.83 alleles per locus, the observed heterozygosity (0.76) was higher than expected heterozygosity (0.39) and majority of variation was within the individuals, indicating high genetic diversity. In the population structures of SSR genotyped data, northern and central-southern *L. indica* accessions were grouped into two clusters with some admixture. Few accessions of *L. indica* showed genetic relatedness with *L. sativa* and *L. serriola*. For all growth and reproductive traits (except seed length, pappus length and thousand seed weight), the genotypic mean square variance was highly significant ($P<0.001$), indicating presence of high genetic variation; broad-sense heritability (h^2B) was also high (>60%). Leaf length, leaf blade width, total and basal branch, node number, plant height, basal and median internode distance, seed per capatilium and thousand seed weight were coupled with high genetic advance as percentage mean (GAM) indicating additive gene effect and selection of these trait based on phenotypic observation is effective for better gain. The duration for bolting, flowering, seed set and seed maturity were linked with moderate GAM and anticipating improvement in productivity via hybridization and selection. Accession number 55 and 8 have shown superior performance for agronomic and morphological characteristics and

can be good material for further research and breeding. The morphological markers were weaker to distinguish accessions.

We also conducted a study to develop an *in vitro* propagation method, for effective breeding through tissue culture. Leaf blade and petiole of *ex vivo* grown plant (acc. 55) was cultured initially on Murashige and Skoog (MS) medium supplemented by various concentrations of 6-benzylaminopurine (BAP) with a fixed concentration (1.2 mg L^{-1}) of indole-3 acetic acid (IAA). Lower BAP concentration (0.5 mg L^{-1}) performed best for shoot regeneration and higher BAP (4 mg L^{-1}) concentration performed best for callus induction. Later, callus were cultured on MS medium supplimented by various concentration of BAP with a fixed concentration (0.5 mg L^{-1}) of IAA; significantly better callus regeneration (98.67%) was obtained on MS medium fortified by 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} IAA. MS medium containing 1 mg L^{-1} IAA was the best for *in vitro* rooting. These plants were successifully adopted in the field. In order to make tissue culture applicable in the phytomedicinal study, lactucin content from *in vitro* and naturally grown tissues and antibacterial activity were investigated. Lactucin was detected in all tissues ranging from 0.2 (tissue cultured leaf) to 2.19 µg g^{-1} (juvenile root) and antibacterial assay of tissue extracts showed antibacterial activity against *Pseudomonas fuscovaginae* (rice pathogen) and *Escheriea coli*. In general, root extracts from both tissues by *in vitro* and naturally grown showed better performance for phytochemical properties and antibacterial activity.

In conclusion, the development of genic-SSR markers and morphological traits not only enabled us to comprehend the genetic diversity, population structure, and agronomic performance for this plant but also helps to gain such information for other *Lactuca* species. Moreover, the established *in vitro* propagation protocol could help for conservation and botanical application of this plant.

Keywords: antibacterial activity; Indian lettuce (*Lactuca indica* L.), *in vitro* propagation, morphological traits; sesquiterpene lactones; SSR marker.

Student Number: 2015-30859

Table of Contents

List of Figures

Supplementary Figures

List of Tables

Supplementary Tables

List of Abbreviations

µg g^{-1}	Microgram per gram
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of molecular variance
BAP	6-benzylaminopurine
BIND	Basal internode distance
BOLT	Duration for bolting
Cm	Centimeter
DAD	Diode Array Detector
EST	Expressed sequence tag
FFD	Duration to first flowering
FMD	Duration to 50 percentage seed maturity
FSD	Duration to first seed set
GA	Genetic advance
GAM	Genetic advance as percentage mean
GCV	Genotypic coefficient of variability
g l^{-1}	Gram per liter
GV	Genotypic variance
h^2B	Broad sense heritability
HPLC	High performance liquid chromatography

IAA	Indole-3 Acetic acid
IBA	Indole-3 butyric acid
LBW	Leaf breadth length
LTL	Leaf tip length
mg L^{-1}	Milligram per millilitre
MIND	Median internode distance
mm^2	Square millimetre
MS	Murashige and Skoog
ng/µl	Nano gram per microliter
PCA	Principal component analysis
PCR	Polymerase chain reaction
PCV	Phenotypic coefficient of variability
PH	Plant height
PIC	Polymorphic information content
PL	Pappus length
POD	Peroxidase
PV	Phenotypic variance
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
SAMPL	Selectively amplified microsatellite polymorphic loci

SL	Seed length
SOD	Superoxide dismutase
SPC	Seed per capitulum
SSR	Simple sequence repeat
STL	Sesquiterpene lactones
TCL	Thin layer chromatography
TLL	Total leaf length
TNN	Total node number
TWS	1000 seed weight
UV	Ultra violet

Introduction

The global population is expected to reach 9 billion by 2050, a significant proportion of which will be urban dwellers, requiring a 70% increase in agricultural productivity (Corvalan et al. 2005; Tilman et al. 2011). To feed these people, the production of high-quality food must increase with reduced input but this accomplishment will be particularly challenging in the face of global environmental change. Plant breeders need to focus on traits with the greatest potential to increase yield particularly to gain more harvest in limited land uses. In addition, agricultural inputs must be reduced to those of insecticides, pesticides, herbicides and fertilizers. Moreover, the cultivated crops have lost genetic diversity due to the influence of human during the process of domestication (Abbo et al. 2014; Zhang et al. 2017). Therefore, crop scientist should look for most significant and heritable agronomic traits in crop wild relatives. Crop wild relatives are the wild plant species that are genetically related to cultivated crops. These plants are less or not influence by human activities and continue to evolve in the wild, developing traits such as biotic and abiotic resistance. These traits of wild plants can cross with domesticated crops to produce new varieties beneficial to human. For proper identification of crop wild relatives, it is very essential to know the background of the wild crop in term of

morphological and genetic basis. Studies on genetic diversity help to identify allele that enables the organism to survive in the existing environment or more diverse habitat (Nei 1987; Smith 1989; Zhang et al. 2017). If the individuals got any variation in its genomic level due to various reasons such as mutation, genetic drift, founder effects and gene flow, then this variation can be expressed in their phenotypes (Vu et al. 2015), understanding the effect of genetic background of mutant phenotype helps to extract the important characters beneficial for human welfare.

The genus *Lactuca* belongs to the tribe Cichoriae (Lactuceae), subfamily Cichorieae of the Asteraceae (Composite), one of the largest plant families (Wei et al. 2017). Cichorieae are mainly distributed over temperate zones in the Northern Hemisphere, in both the old and new worlds (Beharav et al. 2014). The Mediterranean region is considered the center of biodiversity for the *Lactuca* genus and thought to be the probable center of domestication for cultivated lettuce (Beharav et al. 2006; Lebeda et al. 2007). Natural *Lactuca* spp. are widely distributed across the world (Lebeda 1998; Lebeda et al. 2004); reportedly around 100 species with the largest number of autochthonous species (species richness: Asia-51 species, Africa-43 species and Europe-17 species) (Lebeda 1998; Lebeda and Astley 1999; Lebeda et al. 2004). However, only about 22 species have been reported in world gene bank collection (Doležalová et al. 2002). Nevertheless, only a few species such as *L. serriola*, *L. virosa* and *L. saligna* consisting more

than 90% of collections in the International *Lactuca* Database (ILDB), which are mostly of European origin. Moreover, in terms of eco-geographic location Asian, American and African origin comprises around 83% of *Lactuca* species and their collection is very low compared to the European origin in gene bank (Lebeda et al. 2004).

Most of the research in wild *Lactuca* have been carried out for the study of *L. serriola, L. saligna, L. virosa,* and *L. tartarica* and the researcher believe that these species are the important genitors for the lettuce breeding because of crossing behavior and close genetic distance, with cultivated lettuce (Koopman et al. 2001; Lebeda et al. 2009). However, only *L. sativa* L. is the domesticated lettuce in the genus. Nevertheless, a substantial number of Asian *Lactuca* species are yet to explore. In order to make an accurate choice of most promising wild species to further broaden the lettuce gene pool, more research insight in the genetic and biotechnological aspect of wild lettuce is needed, which help for cultivar development and improvement of the trait in domesticated lettuce.

Lactuca indica L., is promising wild lettuce of genus *Lactuca*, which was originated in Africa also (South Africa, Mozambique, Madagascar, Mauritius, Seychelles) (Jeffrey 1966) and widespread in Asian countries probably by Chinese immigrants and relatively common to Indonesia, Malaysia, China, Taiwan, and Japan where it occurs wild and cultivated. Moreover it grows in East

Himalaya (Darjeeling, Arunachal Pradesh), Tibetan plateau, Assam, Burma, North Asia (Siberia, Russia, Far East), and South East Asia (Ohwi 1984); (some information can be obtained in http://www.efloras.org/, http://ecocrop.fao.org,) in Korea, this plant is found in diverse habitat such as roadside, highways, stream banks, grassy areas, anthropogenic and ruderal places. It can be cultivated from the lowlands up to 2000 m altitude.

This plant is annual or perennial, erect, lactiferous herb, with radical rosette when young, up to 2 m tall when flowering. The propagation is by seed, which germinates 3-4 days after sowing, or by root cutting which develops into buds. Seed is usually sown in soil bed and then transplanted in the field. Three to four weeks after germination is the suitable time for transplantation. The plant is edible and used as soup, salad, fermented food (called “kimchi” in Korea), to warp rice and meat while eating. Moreover, it is also used traditionally as herbal medicine to treat fever, cough, cold, and stomach disorders in East-Asian countries including, Korea, China, India, Japan, Indonesia (Facciola 1990). In some areas, the plant is used as fodder to geese and silkworms. Recently many research has carried for application of plant extract in medicine to heal various kind of diseases because of the anti-oxidant, anti-inflammatory, anti-bacterial, digestive, diuretic, necrotic, and sedative properties of the plant (Bown 1995; Ha et al. 2017; Kim et al. 2007; Seo et al. 2009; Wang et al. 2003b). Furthermore, the plant is resistant to downy mildew (Van Treuren et al. 2013). In addition,

significant amount of lactucin, a highly important sesquiterpene lactone, enrichment of biotic and abiotic stress responsive gene reported (Ha et al. 2017), different amount of proximate compositions such as minerals, reducing sugar, free amino acids, organic acids, vitamin C (Kim et al. 2012), and higher amount of phenolic and flavonoids have reported (Kim and Yoon 2014). Because of these properties, it raised the interest of the crop scientist. Moreover, the collection of germplasm from the diverse habitat enriches the poor collection status of *L. indica* in the ILDB, where only eight accessions of *L. indica* are reported. Among them, three accessions LAC 549, CGN 14316, and CGN20713 were china origin, one accession CGN14312 from Indonesia, one accession LAC 1200 from Japan, and other three accessions CGN13392, CGN13393, and PI236396 are unknown in origin.

Understanding the level of genetic diversity would help to develop new and improved cultivar with desirable traits both for farmers and breeders prospective (Mondini et al. 2009; Upadhyaya et al. 2008). Molecular markers, are one of the advance tools to study genetic diversity in germplasm collection and also useful to compare the phylogenetic relationship of a wild plant with respect to the cultivated crop. Molecular markers have been used to study genetic relationship in genus *Lactuca* including *L. indica* (El-Esawi and Sammour 2014; Kesseli et al. 1991; Koopman et al. 1998; Lebeda et al. 2009; Witsenboer et al. 1997). However, single or few accessions were used to compare the phylogenetic

position with other species including *L. sativa*. Furthermore, they have used marker developed in the primary gene pool of lettuce rather than *L. indica* genome. Therefore, a molecular marker that can amplify *L. indica* genome and across *Lactuca* genus are required to further explore and utilize *L. indica* more effectively.

Recently, transcriptome sequencing data for *L. indica* is available (Ha et al. 2017). These data can be mined to genic simple sequence repeat markers (SSR). These genic- SSRs are useful as molecular markers because their development is inexpensive, they represent transcribed genes (developed from transcript) and a putative function can often be deduced by a homology search. These genic-SSR are useful for studying the diversity in natural populations or germplasm collections. Furthermore, these markers have high amplification, transferability to related species, more stable for evolutionary study and comparative mapping and could prove useful for marker-assisted selection especially regarding the gene responsible for phenotypic traits (Varshney et al. 2005).

Agronomic traits such as yield can be affected by a large number of environmental variables. Finding the important traits associated with yield and yield component has greater value in agronomy. Study of agro-morphological variation helps to identify the important traits controlling the yield and yield

components by selecting difficult or expensive and low heritable components. Moreover, the marker-assisted selection helps to identify the gene controlling the concerned traits. By adopting the proper gene engineering approach, an unwanted gene that adversely affects the yield can be removed and clone for the mass propagation using tissue culture. Furthermore, tissue culture provides a continuous supply of materials without any seasonal limitation, which helps to decrease the dependency on the natural sources for extraction of valuable drugs, hence help to conserve medicinal plant (*L. indica*) resource for next generation (Chen et al. 2016; Wang et al. 2017).

As mentioned earlier, this study adds three important aspects of the lettuce resource. First, identify the possibility of this wild plant in lettuce breeding, which gives immense information about the lettuce gene and provides the genetic relationship between other lettuce. Moreover, it gives ideas to the researcher to develop new cultivar by exchanging the important traits with each other. The second aspect of *L. indica* research is the possibility of mass propagation to lower the overharvesting for the medicinal purpose in the natural habitat and cope with increasing lettuce demand. Thus, in order to meet the purpose, this study has three objectives as follows-

1. To develop genic-SSR marker from *L. indica* transcriptome sequence and apply these markers for studying genetic diversity of *L. indica*, marker transferability and genetic relatedness to other *Lactuca* species.

2. To characterize the morphological variation, agronomic performance and genetic diversity using agro-morphological traits of *L. indica* accessions.
3. And to perform tissue culture for mass propagation, comparative study of lactucin and antibacterial activity of tissue extracts collected from *in vitro* and natural environment.

To meet these objectives, this dissertation is divided into three chapters. Chapter-I deals with the development of SSR marker and its application based on the *L. indica*. Moreover, the developed marker is used for the study of genetic diversity and identifying the relation between other *Lactuca* species. Chapter-II deals with the genetic diversity and agronomical performance for agro-morphological traits. And in Chapter-III *in vitro* propagation protocol is established, lactucin from *in vitro* and naturally grown tissue extract is quantified and the antimicrobial test is performed.

Literature reviews

Genetic diversity of wild plant for crop development

Plant genetic resources comprise all agricultural crops and their wild relatives of valuable traits. Cultivated plants have experienced a dramatic loss of genetic diversity due to anthropogenic influence (Chen et al. 2014; Zhang et al. 2017). The potential of wild *Lactuca* species to be used in lettuce breeding is being demonstrated by means of classical biology and modern approaches. The study of their diversity has been a subject of theoretical research and practical application since past 30 years (Lebeda et al. 2007; Mou 2008; Ryder 1999). The wild relatives have become an important source for lettuce breeding by serving as donor resistant genes against biotic and abiotic stress, as well as the gene for improving physiological and quality traits (Beharav et al. 2006; Beharav et al. 2014; Lebeda et al. 2009; Lebeda et al. 2007). Genetic variation in a population allows species to adjust to different environmental conditions. In addition, genetic diversity and variation studies are vital for providing information for propagation, taxonomy, disease resistance, and breeding programs as well as conservation and utilization of *Lactuca* genetic resources. The genetic diversity study along with

the relatedness measure can elucidate how wild and domesticated species share and maintain the important traits. Moreover, it provides the indication of the potential hybridization improvement of the important trait. The relationship between cultivated and wild lettuce and the implication of relationship of gene flow from cultivated to the wild was analyzed (De Vries et al. 1994). In *Lactuca* genus, only a few species such as *L. sativa*, *L. serriola* f. *serriola*, *L. serriola* f. *integrifolia*, *L. saligna*, *L. aculeata* and *L. virosa* have used for the lettuce improvement (Lebeda et al. 2009).

Simple sequence repeat (SSR) markers

During the past decades, molecular techniques have become the most effective tool to evaluate genetic variation in the population. Molecular markers are DNA based genetic markers that can detect DNA polymorphism at the level of specific loci or whole genome (Varshney et al. 2006). These markers are based on DNA itself, and not the gene product. Because of this reason, molecular markers have gained importance for new germplasm characterization. Moreover, these markers are unlimited in number in the genome, do not vary among the tissue types,

developmental stage or the environmental influence and produce quality information (Govindaraj et al. 2015).

Simple sequence repeats (SSR) markers are of choice among different types of molecular markers because these markers are tendon repeats of short nucleotide (1-6 bases), randomly spread in eukaryotic genome, co-dominance, high reproducibility, polymorphic, very little DNA is required, cheap and easy to handle. Moreover, genic SSRs have some intrinsic advantages as they are present in expressed regions of the genome and expected high amplification and transferability (Varshney et al. 2005).

There is very limited information for SSR marker developed and utilization in genomic research for genus *Lactuca*. Previously Simko (2008) developed expressed sequence tag simple sequence repeats (EST-SSR) from the *L. sativa* and *L. serriola* unigenes and marker polymorphisms were tested for cultivated lettuce including three wild lettuces *L. serriola, L. saligna,* and *L. virosa*. Thus, developed EST-SSR was used for analyzing population structure of *L. sativa* cultivars. Based on their findings, they have concluded that EST-SSRs were highly effective for marker transferability and genetic diversity study and gave the idea for the possible implication of this EST-SSR for linkage map construction, cultivar genotyping, population structure analysis, association studies and phylogenetic study in *Lactuca* species. In the year 2010, EST-SSR

was developed from the expressed sequence tags (ESTs) of five *Lactuca* species, *L. serriola*, *L. sativa, L. perennis, L. virosa*, and *L. saligna* and used to study marker transferability across species (*L. sativa* and *L. perennis*) and across genera (*Helianthus annuus*), their result showed significant amount of cross-species transferability across species and genera. Moreover, EST-SSRs markers were useful for discriminating *L. serriola* biotypes and other species of genus *Lactuca* (Riar et al. 2010). Later, Rauscher and Simko (2013), developed genomic SSR marker in *Lactuca sativa* and used to amplify the genomic DNA of *L. sativa* along with three wild lettuce *L. serriola, L. saligna*, and *L. virosa* (these species belongs to the primary gene pool of cultivated lettuce). Further these SSR markers were used to construct linkage map for *L. sativa* (cv. Salinas) × *L. serriola* (accession UC96US23) and genetic relationship study among *L. sativa* accessions (Rauscher and Simko 2013) they also concluded that genomic-SSR can be used for cultivar fingerprinting, construction of integrated molecular linkage maps, and mapping genes of interest. Recently, comprehensive transcriptome sequencing and functional annotation of *L. indica* is available (Ha et al. 2017). De novo transcriptome assembly yielded 73,300 unigenes from 127 million reads based on 12.9 GB of data. They have identified 1685 transcriptome factor genes belonging to 53 families and 8830 SSR loci based on transcripts. They also identified 23 key enzymes in the mevalonate and non-mevalonate pathways of terpenoid biosynthesis. This transcriptomic data provides a valuable

genetic resource for further research and breeding studies of this traditional medicinal herb.

Morphological variation and agronomic performance ability

Morphology indicates the physical appearance of individuals, diversity of which in any dimension can be used to differentiate group and individual population from each other based on their visual effect on qualitative as well as quantitative characters. The variations in qualitative characters such as structure, shape, architectural arrangement or orientation of the plant are discontinuous variations and are controlled by single gene loci and less influenced by environmental factors. However, the variation in quantitative characters such as plant height, node number, flowering time, seed weight etc. are continuous variation which is governed by multiple gene systems and highly influenced by environmental factors.

Wide variation for morphological and developmental traits generates fundamental knowledge on domestication and provide very important information for plant breeders for quantifying genetic variation among the traits and helps to select desirable phenotypes (Bhattarai et al. 2016). *Lactuca* species generally

have wide geographical distribution encompassing the wide range of environmental conditions (Doležalová et al. 2002; Lebeda et al. 2009; Oliya et al. 2018) and showed variation in phenology, development, and morphology (Doležalová et al. 2002). Morphological data (such as stem, leaves, inflorescence, achenes) were used for correct taxonomical identification of individual *Lactuca* spp (Dolezalová et al. 2003). The descriptor of morphological characters of *Lactuca* genetic resources has published (Doležalová et al. 2002; Kristkova et al. 2008) which serve as a tool for correct taxonomic determination and help to perform both interspecific and intraspecific morphological variation study. Among the different types of variation, leaf shape variability is the most accounted and which ranges from a different degree of lobing to entire (Doležalová et al. 2002; Lindqvist 1958). *L. serriola*, accessions from Czech Republic, Germany, Netherlands and United Kingdom showed that leaf shape variation highly influences by eco-geographical conditions as the samples from the Czech Republic, only three types of leaf shape pinnatipart, pinnatifid and pinnatisect were observed (absent of entire types of leaves). Among them, pinnatisect were dominated, however, in the United Kingdom, entire types of leaf dominated in the sample (Lebeda et al.2009).

De Vries and Van Raamsdonk (1994) used numerical morphological approach to compare morphological variation with accessions of *L. saligna*, *L. serriola*, *L. virosa* in order to find the intraspecific and interspecific variation.

Principal component analysis distinguished four groups corresponding to four species. Moreover, intraspecific variation (eg. Color, leaf shape) within *L. sativa* and *L. serriola* was largest. Beharav et al. (2008) collected *L. saligna* L. from 41 localities representing different climatic and edaphic environment through Israel. In their study, individual populations were varied in size and morphological uniformity/heterogeneity. Based on their research they gave the idea that, this wild lettuce could carry morphological and genetic variation and could be exploited in lettuce breeding. Beharav et al. (2010) studied morphological variation characterizing different qualitative and quantitive traits in *L. aculeata* Boiss. Et Ky collected from Northeastern Israel from natural habitat. Israeli populations of *L. aculeata* did not exhibit broad morphological variability, however, high variation was observed in developmental stages such as duration for bolting, flowering, seed maturity and first ripe achene. They also concluded that the unique collection of wild lettuce *L. aculeata* may carry a novel source of genetic variation for a wide range of trait and thus, should be interesting for exploitation in lettuce breeding. Later, the morphological comparison and possibility of intra-species crossing in wild *Lactuca* spp. *L. serriola* and *L. aceluata* were performed and the intermediate characters of the putative natural hybrid were discussed (Lebeda et al. 2012). Furthermore, a significant difference in developmental stages between *L. serriola* and *L. aculeata* originating in a

comparable eco-geographical condition were observed during cultivation in greenhouse condition (Lebeda et al. 2012).

Flowering, one of the important trait for commercial vegetable, is mediated by environmental factors (such as light period, temperature, elevation and etc.) stage of growth (Silva et al. 1999) resource availability (nutrient and water) (Rathcke and Lacey 1985) and biotic factors including pollinators, seed dispersers and floral pathogens (Elzinga et al. 2007). Duration for bolting (transition between vegetative and reproductive stage) and flowering are an important agronomic trait for quality leaf production in the lettuce because at flowering time lettuce produce secondary metabolites such as sesquiterpene lactone which causes the undesirable test to lettuce thereby reduce the lettuce market making plant unsaleable (Han et al. 2016). However, early bolting/flowering genotypes can be used to speed up generation time in genetic and growth studies. Both flowering and bolting are inherited and genetically controlled by a specific gene, however, each gene has an independent function (Han et al. 2016; Silva et al. 1999).

Plant height, node number, internode distance and stem diameter are a crucial component of horticultural crops. Plant height is critical to species' competitive ability and survival and is related to many important functional traits (Leck et al. 2008). It is a major determination of plants ability to compete for

light and show correlation with other traits such as node number, number of branches, leaf nitrogen ratio, photosynthesis, flowering, and stem density or stem diameter (Falster and Westoby 2003). These traits are associated with the yield (Leck et al. 2008). Stem diameter and density have value in breeding because low stem density allows for high relative growth (Poorter et al. 2008) and thicker stem diameter contributes to resistance to breakage and pathogen attack (Zanne et al. 2009). In herbaceous species, taller species flower later than the shorter one (Dahlgren et al. 2007; Du and Qi 2010; Moles et al. 2009; Sun and Frelich 2011).

Genetic diversity of *L. indica* based on molecular and morphological marker analysis

Few attempts have made for genetic study of *L. indica* using molecular markers. Kesseli et al. (1991) used restriction fragment length polymorphism (RFLP) marker for studying genetic diversity among *Lactuca* species. They used 67 accessions of *Lactuca* including *L. sativa* (44 accessions), *L. serriola* (4 accessions), *L. saligna* (5 accessions) and *L. indica* (1 accession). In their phylogenetic study, *L. indica* was far from *L. sativa* and made the conclusion that, *L. indica* is beyond the lettuce gene pool. On the same set of experiment, Hill et

al. (1996) performed genetic relatedness study using amplified fragment length polymorphism marker (AFLP) and found similar result with Kesseli et al. (1991). Later, Koopman et al. (1998) used internal transcribed spacer (ITS-1) for studying phylogenetic relationship among 97 accessions representing 23 species of *Lactuca* and related genera including *L. sativa* (7 acc.), *L. serriola* (6 acc.) *L. indica* (2 acc.). In their phylogenetic analysis, a clade with *L. sativa* and *L. serriola* were the members of the primary gene pool. *L. indica* was not the part of lettuce gene pool, however, the position of *L. indica* was unclear as its position varied in two different method of phylogenetic tree. Witsenboer et al. (1997) used selectively amplified microsatellite polymorphic locus (SAMPL) analysis, in morphologically diverse line of *L. sativa* (44 acc.) and five wild lettuces that were examined in the other previous study (Kesseli et al.1991; Hill et al. 1996). In their phylogenetic tree of Witsenboer et al. (1997), the position of *L. indica* was in between *L. virosa* and *L. perrinnis*, but far from *L. sativa* (Witsenboer et al. 1997). Recently, using chloroplast gene sequences (ndhF and trnL-F), Wei et al. (2017) analyzed the phylogenetic relation between 89 individuals belonging to 48 species including *L. sativa* (3 acc.), *L. serriola* (3 acc.) and *L. indica* (4 acc.). In their phylogenetic reconstruction the position of *L. indica* was next to crop clade comprising of *L. sativa* and *L. serriola.* The reported studied used single or few accessions of *L. indica* and the molecular markers developed for other *Lactuca*

species rather than *L. indica* genome. Molecular marker used in *Lactuca* genetic study were reviewed in previous studies (El-Esawi 2015; Lebeda et al 2009)

To the best of our knowledge, scientific study for genetic diversity of *L. indica* using morphological markers are lacking. However, few studies were conducted for morphological characterization of this species. This species was not crossable with the species in lettuce gene pool, nevertheless, with *L. laciniata*, *L. graminifolia*, *L. floridana* and *L. spicata*, this plant produced self-sterile or partly fertile or fertile hybrid plants (Thompson et al. 1941). Moreover, *L. indica* share some of the morphological characters to other *Lactuca* species for instance, as in *L. sativa* and other lettuce including *L. serriola*, this plant showed variation in leaf shape ranging from entire leave to strongly pinnately lobed leaves and variation in anthocyanin distribution. Other reported morphological characteristics of *L. indica* are: it flowers under short-day conditions, produces numerous ligules in the head, have light purple anther tube, broadly winged achene with short beak of pale color and showed late flowering in greenhouse experiment (Doležalová et al. 2002). Despite the use of molecular and morphological marker, this plant produced viable callus through somatic hybridization with *L. sativa* (Mizutani et al. 1989) and also showed resistance to downy mildew (van Treuren et al. 2011).

Secondary metabolites (sesquiterpene lactones) quantification in *L. indica*

Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years. Wounding leaves or stems of *Lactuca* species releases a milky latex, lactucarium, which is the rich source of Sesquiterpene lactones (SLs). SLs are class of chemical compounds, built from three isoprene units and contain the lactone ring. SLs found in plant are diverse remarkably diverse in term of their structure, properties and purposed functions. Among the compositae, at least 500 different members of SLs have been described (Sessa et al. 200). Lactucin and lactucin-type guaianolides, as well as melampolide-type germacranolide lactuside-A, have been characterized from the species in tribe Lactuceae including *Lactuca, Cichorium, Crepidiastrum, Leontodon, Mycelis, Picris, Sonchus* and *Youngia* (Michalska et al. 2009; Wang et al. 2010; Zidorn 2008).

By using spectral data and X-ray diffraction analysis, several SLs such as 9α-hydroxyzaluzanin C, 9α-hydroxy11, 13dihydrozaluzanin C, lactucopicriside, lactuside A and lactuside B, macrocliniside A, glucozaluzanin C, 11,13adihydroglucozaluzanin C, 11β,13-dihydrolactucin and dihydrosantamarin were identified on the roots of *L. laciniata* (= *L. indica)* (Nishimura et al. 1986).

Michalska et al. (2009) using ultra violet-high performance chromatography (UV–HPLC) and thin layer chromatography (TLC) comparisons, eight different types of STLs in leaves and root of eleven *Lactuca* taxa were characterized. Eleven taxa showed the quantitative difference in the compound identified. Retention times and on-line DAD UV spectra with absorption maxima at 260 nm (lactucin-type guaianolides, 8-deoxylactucin, jacquinelin, crepidiaside B, lactucin, 11b, 13-dihydrolactucin and lactucopicrin), 235 nm (lactuside A) and 210 nm (all compounds) were assessed as criteria for the identification of the observed peaks with the reference substances. Among them, six different types of STLs, 8-deoxylactucin, jacquinelin, crepidiaside B, lactucopicrin and vernoflexuoside = glucozaluzanin C and lactuside A were identified in *L. indica*. Among the six identified SLs all were present in the root, however, lactucopricin, Jacqueline, and Lactuside A were absent in leaf tissue. The most notable is, they did not identify lactucin both in leaf and root of *L. indica*. Recently, using high-performance liquid Chromatography (HPLC), various concentrations (1.9 $\mu g\, g^{-1}$ to 98.7 $\mu g\, g^{-1}$) of lactucin is characterized in the flowering time leaf of *L. indica* collected in diverse habitat and eco-geographic location of South Korea (Ha et al. 2017). Besides SLs several phenolic and flavonoids have reported in the areal part of *L. indica* (Kaneta et al. 1978; Kim and Yoon 2014; Kim et al. 2008; Park et al. 2014; Wang et al. 2003a). The phenolic compound is a key compound of antioxidant activity (Cartea et al. 2010). Many antioxidant enzymes such as

superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) against ROS (reactive oxygen species) have reported (Zhu et al. 2001) which have several health benefits.

Lactuca indica in medicinal research

SLs contribute bitterness in *Lactuca* spp. and chicory. Since ancient times, *Lactuca* species has been well known as a dietary and medicinal plant and is famous for its dietary leaves and salad worldwide (Food and agriculture organization available at http://faostat.fao.org). It is likely that this bitter taste protects the plants from mammalian herbivores. Moreover, the lactucin-type guaianolides 8-deoxylactucin, jacquinelin, and lactucopicrin have been shown to possess an antifeedant activity against insect pests (Michalska et al. 2009). SLs found in *Lactuca* species represents a good target for genetic manipulation leading to an enhanced phytoalexin response for the disease resistance (Michalska et al. 2009).

An extract from *L. indica* possessed significant free radical scavenging activity, effectively protecting φx174 supercoiled DNA against strand cleavage and reducing oxidative stress in human promyelocytic leukemia HL-60 cells.

Moreover, the extract showed nitric oxide production inhibition and mRNA expression of inducible nitric oxide synthase, at a dosage of 100 μg mL^{-1}, in LPS stimulated macrophage RAW264.7 cells (Wang et al. 2003). This finding suggests that *L. indica* can be used as food additive for human health care. Terpenoids and phenolics isolated from the areal part of this plant showed cytotoxicity against four human tumor cell lines *in vitro* using a Sulforhodamin B bioassay (Kim et al. 2008). The methanol extract of leaf showed the highest cytotoxicity (IC50 113.84 μg mL^{-1}) against human breast adenocarcinoma cell (MCF-7) and phenolic content showed antioxidant properties (Park et al. 2014). STLs lactucin C and lactucaside showed significant antidiabetic activity (Hou et al. 2003). Quinic acid derivatives and phenylpropanoid derivative (di-E-caffeoyl-meso-tartaric acid) showed hepatoprotective activity against Hepatitis B virus (HBV) (Kim et al. 2007; Kim et al. 2010). These compounds were isolated from the methanol extract of this plant. To the best of our knowledge, no attempts have been made to test its extracts against disease in plants such as antibacterial properties and its test regarding the bacteria of major crops and commercial vegetables.

Tissue culture for commercial cultivation and synthesis of secondary product

Scientist such as botanist, ethnobotanists, ethnopharmacologists are interested in the medicinal plants as these plants are important source for several health benefit and drug discovery (Balunas and Kinghorn 2005). An alarming note is that very few amounts of plants are cultivated threatening the possible wiping out the species. Moreover, in the developing countries, people ruinous harvest medicinal plants from the wild source for ethnobotanical activities without considering the future. Moreover, phytochemical industries prefer not to produce the raw material for the extraction rather outsourcing from the different collectors. Such high dependency to third-party supplier of raw material have seriously diminished medicinal plants in their natural habitat disturbing the plant diversity with little or no regard to the future (Abbad et al. 2011; Anis and Ahmad 2016; Salgotra and Gupta 2016). The plant tissue culture technique provides a valuable tool for conservation and their practical application in commercial scale. Tissue culture methods have proved successful in rescuing selected lettuce genotypes and producing seeds in a disease-free environment (Jenni et al. 2006).

The plant tissue culture provides a valuable tool for manufacturing secondary metabolites. Over past decades' tissue culture has emerged as an

alternative of whole plant cultivation in the production of valuable secondary metabolites and these metabolites has been recognized as obtained in their parental plant (DiCosmo and Misawa 1995). Many research carried out for the *in vitro* production of secondary metabolites are listed and optimized results are highlighted (Chavan et al. 2018; Wang et al. 2017). Based on these reviews, most of the research on tissue, and organ culture research mainly focuses on optimization of culture conditions, composition comparison, commercial cultivation, transgenic technology, and genetic stability.

Chavan et al. (2013) assessed the different extracts of three lantern flower species viz. *Ceropegia spiralis*, *C. panchganiensis* and *C. evansii* and revealed the presence of phenolics, flavonoids and antioxidant compounds in various organs of these species. In the subsequent study by Chavan et al. (2014), significantly higher amount of phenolics, flavonoids and antioxidants in indirect organogenesis derived plant than direct organogenesis derived and field grown plant was detected. Recently several research held in different plant showed the higher concentration of secondary metabolites have detected in tissue cultured plants (Bhattacharyya et al. 2017; Bose et al. 2016; Paul et al. 2017). The concentration of secondary metabolites also depends on the type of tissue, culture, and the media used. Moreover, tissue cultured plant extract has applied for antimicrobial activities (Al Khateeb et al. 2012; Kumari et al. 2016).

Adventitious roots culture of *Panax ginseng* and *Echinacea purpure* has reached the scale of 1−10 KL (Jeong et al. 2009; Murthy et al. 2014) and somatic embryos of *Acanthopanax senticosus* reached 500 L (Paek et al. 2014). Hairy root culture of witloof chicory (*C. intybus* L. cv. Lucknow local), obtained by transformation with Agrobacterium rhizogenes produce secondary compounds coumarins, esculin and esculetin (Bais et al. 1999). A transformed root culture of *Cichorium intybus* L. (Compositae) produce SLs germacrene types-Lactucopicrin, 8-desoxylactucin, and three SLs glycosides: crepidiaside B, sonchuside A and ixerisoside D; the yield of 8-desoxylactucin was 0.03 g^{-1} (Malarz et al. 2002). Similarly, a higher amount of inulin was extracted from the transgenic hairy root of *Cichorium* spp. (Hanafy et al. 2018).

In vitro propagation in lettuce

Axillary buds of *L. sativa* was tissue cultured on MS medium supplimented by various concentrations of BAP, IAA and kinetin, MS + 1.0 or 2.0 mg L^{-1} kinetin and 6.4 mg L^{-1} IAA performed better to promote shoot growth (Pink and Carter 1986). Somatic embryos have been regenerated in seven genotypes of *L. sativa* using leaf, stem-internode and petiole of (Seabrook and Douglass 2002). Mohebodini et al. (2011) investigated callus induction and direct shoot

regeneration of lettuce using cotyledon explant and highest number of direct shoot regeneration was obtained at low BAP concentrations and the effect of explant for callus induction was a genotype-dependent. Recently, cotyledon explant of *L. sativa* was used for multiple shoot organogenesis where, MS medium supplemented with 200 mg L^{-1} of activated charcoal, with 3% sucrose, 10 mg L^{-1} BAP and 0.5 mg L^{-1} NAA performed better response for multiple shoot organogenesis (Armas et al. 2017).

Among the wild lettuce, *in vitro* propagation have reported only in *Launaea taraxacifolia* (Willd.) Amin ex C. Jeffrey (Sakpere et al. 2011; Obembe et al. (2017). For this species, Sakpere et al. (2011) used stem and leaf explant on MS medium with varying concentration of 2,4- dichorophenoxyacetic acid (2,4-D), leaf explants performed better response for regeneration of shoots than stem explant. Obembe et al. (2017) used nodal segment for various concentration of auxin, NAA; with BAP and kinetin on MS basal medium for morphogenic response and found that BAP performed better response for multiple shoot formation. To the best our knowledge, no studies have conducted for *in vitro* propagation of *L. indica.*

References

Abbad A, Belaqziz R, Bekkouche K, Markouk M (2011) Influence of temperature and water potential on laboratory germination of two Moroccan endemic thymes: Thymus maroccanus Ball. and Thymus broussonetii Boiss. Afr J Agr Res 6:4740-4745

Abbo S, van-Oss RP, Gopher A, Saranga Y, Ofner I, Peleg Z (2014) Plant domestication versus crop evolution: a conceptual framework for cereals and grain legumes. Trends Plant Sci 19:351-360

Al Khateeb W, Hussein E, Qouta L, Alu'datt M, Al-Shara B, Abu-Zaiton A (2012) *In vitro* propagation and characterization of phenolic content along with antioxidant and antimicrobial activities of *Cichorium pumilum* Jacq. Plant Cell, Tiss. Org Culture (PCTOC) 110:103-110

Anis M, Ahmad N (2016) Plant Tissue Culture: Propagation, Conservation and Crop Improvement. Springer, Nature, Singapore

Bais H, George J, Ravishankar G (1999) Influence of polyamines on growth of hairy root cultures of witloof chicory (*Cichorium intybus* L. cv. Lucknow

Local) and formation of coumarins. Journal of plant growth regulation 18:33-37

Balunas MJ, Kinghorn AD (2005) Drug discovery from medicinal plants Life sciences 78:431-441

Beharav A, Lewinsohn D, Lebeda A, Nevo E (2006) New wild *Lactuca* genetic resources with resistance against Bremia lactucae. Genet Resour Crop Evol 53:467-474

Beharav A, Ben-David R, Dolezalova I, Lebeda A (2010) Eco-geographical distribution of *Lactuca aculeata* natural populations in northeastern Israel. Genet Resour Crop Evol 57:679-686 doi:10.1007/s10722-009-9503-6

Beharav A, Ochoa O, Michelmore R (2014) Resistance in natural populations of three wild *Lactuca* species from Israel to highly virulent Californian isolates of Bremia lactucae. Genet Resour Crop Evol 61:603-609

Bhattacharyya P, Kumaria S, Bose B, Paul P, Tandon P (2017) Evaluation of genetic stability and analysis of phytomedicinal potential in micropropagated plants of *Rumex nepalensis*–A medicinally important source of pharmaceutical biomolecules. Journal of Applied Research on Medicinal and Aromatic Plants 6:80-91

Bhattarai K, Louws FJ, Williamson JD, Panthee DR (2016) Diversity analysis of tomato genotypes based on morphological traits with commercial breeding significance for fresh market production in eastern USA. Australian Journal of Crop Science 10:1098

Bose B, Kumaria S, Choudhury H, Tandon P (2016) Assessment of genetic homogeneity and analysis of phytomedicinal potential in micropropagated plants of *Nardostachys jatamansi*, a critically endangered, medicinal plant of alpine Himalayas. Plant Cell, Tissue and Organ Culture (PCTOC) 124:331-349

Bown D (1995) The encyclopedia of herbs & their uses. Dorling Kindersley: London, U. K

Cartea ME, Francisco M, Soengas P, Velasco P (2010) Phenolic compounds in *Brassica* vegetables. Molecules 16:251-280

Chavan J, Gaikwad N, Dixit G, Yadav S, Bapat V (2018) Biotechnological interventions for propagation, conservation and improvement of 'Lantern Flowers'(*Ceropegia* spp.). S Afr J Bot 114:192-216

Chavan J, Gaikwad N, Umdale S, Kshirsagar P, Bhat K, Yadav S (2014) Efficiency of direct and indirect shoot organogenesis, molecular profiling,

secondary metabolite production and antioxidant activity of micropropagated *Ceropegia santapaui*. Plant growth regulation 72:1-15

Chavan JJ, Gaikwad NB, Kshirsagar PR, Dixit GB (2013) Total phenolics, flavonoids and antioxidant properties of three Ceropegia species from Western Ghats of India. South African journal of botany 88:273-277

Chave J, Coomes D, Jansen S, Lewis SL, Swenson NG, Zanne AE (2009). Towards a worldwide wood economics spectrum. Ecology letters, 12(4), 351-366

Chen C, Li P, Wang R-H, Schaal BA, Fu C-X (2014) The population genetics of cultivation: domestication of a traditional Chinese medicine, Scrophularia ningpoensis Hemsl.(Scrophulariaceae) PLoS One 9:e105064

Chen S-L, Yu H, Luo H-M, Wu Q, Li C-F, Steinmetz A (2016) Conservation and sustainable use of medicinal plants: problems, progress, and prospects. Chin Med 11:37

Corvalan C, Hales S, McMichael AJ (2005) Ecosystems and human well-being: health synthesis. World health organization

Dahlgren JP, von Zeipel H, Ehrlén J (2007) Variation in vegetative and flowering phenology in a forest herb caused by environmental heterogeneity. American Journal of Botany 94:1570-1576

De Vries FF, Van der Meijden R, Brandenburg WA (1994) Botanical Files on Lettuce (*Lactuca sativa*). On the chance for gene flow between wild and cultivated Lettuce (*Lactuca sativa* L. including *L. serriola* L., Compositae) and the generalized implications for risk-assessments on genetically modified plants. Gorteria-Supplement 2:1-44

De Vries I, Van Raamsdonk L (1994) Numerical morphological analysis of lettuce cultivars and species (Lactuca sect. *Lactuca*, Asteraceae). Plant Syst Evol 193:125-141

DiCosmo F, Misawa M (1995) Plant cell and tissue culture: alternatives for metabolite production. Biotechnology advances 13:425-453

Doležalová I, Křístková E, Lebeda A, Vinter V (2002) Description of morphological characters of wild *Lactuca* L. spp. genetic resources (English-Czech version). Hort Sci(Prague) 29:56-83

Dolezalová I, Lebeda A, Dziechciarková M, Kristkova E, Astley D, Van de Wiel C (2003) Relationships among morphological characters, isozymes

polymorphism and DNA variability-the impact on *Lactuca* germplasm taxonomy. Czech Journal of Genetics and Plant Breeding 39:59-67

Du G, Qi W (2010) Trade-offs between flowering time, plant height, and seed size within and across 11 communities of a QingHai-Tibetan flora. Plant Ecology 209:321-333

El-Esawi MA, Sammour R (2014) Karyological and Phylogenetic Studies in the Genus *Lactuca* L. (Asteraceae). Cytologia 79:269-275 doi:10.1508/cytologia.79.269

Elzinga JA, Atlan A, Biere A, Gigord L, Weis AE, Bernasconi G (2007) Time after time: flowering phenology and biotic interactions Trends in Ecology & Evolution 22:432-439

Facciola S (1990) Cornucopia: a source book of edible plants.

Falster DS, Westoby M (2003) Plant height and evolutionary games. Trends in Ecology & Evolution 18:337-343

Govindaraj M, Vetriventhan M, Srinivasan M (2015) Importance of genetic diversity assessment in crop plants and its recent advances: an overview of its analytical perspectives. Genetics research international 2015

Ha J, Lee T, Kim MY, Oliya BK, Gwag J-G, Lee Y-H, Lee S-h (2017) Comprehensive transcriptome analysis of *Lactuca indica*, a traditional medicinal wild plant. Mol Breed 37:112 doi:10.1007/s11032-017-0711-z

Han Y et al. (2016) MADS-box genes and gibberellins regulate bolting in Lettuce (*Lactuca sativa* L. Frontiers in plant science 7:1889

Hanafy MS, Asker MS, El-Shabrawi H, Matter MA (2018) Agrobacterium rhizogenes-mediated genetic transformation in *Cichorium* spp.: hairy root production, inulin and total phenolic compounds analysis. The Journal of Horticultural Science and Biotechnology:1-9

Hill M, Witsenboer H, Zabeau M, Vos P, Kesseli R, Michelmore R (1996) PCR-based fingerprinting using AFLPs as a tool for studying genetic relationships in *Lactuca spp*. Theor Appl Genet 93:1202-1210. doi:10.1007/s11032-017-0711-z

Hou C-C, Lin S-J, Cheng J-T, Hsu F-L (2003) Antidiabetic Dimeric Guianolides and a Lignan Glycoside from *Lactuca indica*. Journal of natural products 66:625-629

Jeffrey C (1966) Notes on the Compositae: I. The Cichorieae in EastTropical Africa. Kew Bull 18:427–486

Jenni S, Loukili F, Ehsani Moghaddam B (2006) *In vitro* culture response of apical and axillary shoot-tips excised from crisphead lettuce cores depends on head maturity, not storage time. In Vitro Cell Dev Biol Plant 42:274-277

Jeong C, Murthy H, Hahn E, Lee H, Paek K (2009) Inoculum size and auxin concentration influence the growth of adventitious roots and accumulation of ginsenosides in suspension cultures of ginseng (*Panax ginseng* CA Meyer*).* Acta physiologiae plantarum 31:219-222

Kaneta M, Hikichi H, Endo S, Sugiyama N (1978) Identification of flavones in sixteen Compositae species. Agricultural and Biological Chemistry 42:475-477

Kesseli R, Ochoa O, Michelmore R (1991) Variation at Rflp Loci in *Lactuca* Spp and Origin of Cultivated Lettuce (*L. Sativa*). Genome 34:430-436

Kim J-M, Kim J-N, Lee K-S, Shin S-R, Yoon K-Y (2012) Comparison of physicochemical properties of wild and cultivated *Lactuca indica.* Journal of the Korean Society of Food Science and Nutrition 41:526-532

Kim JM, Yoon KY (2014) Comparison of polyphenol contents, antioxidant, and anti-inflammatory activities of wild and cultivated *Lactuca indica.* Horticulture, Environment, and Biotechnology 55:248-255

Kim KH, Kim YH, Lee KR (2007) Isolation of quinic acid derivatives and flavonoids from the aerial parts of *Lactuca indica* L. and their hepatoprotective activity *in vitro*. Bioorganic & medicinal chemistry letters 17:6739-6743

Kim KH, Kim YH, Lee KR (2010) Isolation of hepatoprotective phenylpropanoid from *Lactuca indica*. Nat Prod Sci 16:6-9

Kim KH, Lee KH, Choi SU, Kim YH, Lee KR (2008) Terpene and phenolic constituents of *Lactuca indica* L. Archives of pharmacal research 31:983-988

Koopman WJM, Guetta E, van de Wiel CCM, Vosman B, van den Berg RG (1998) Phylogenetic relationships among *Lactuca* (Asteraceae) species and related genera based on ITS-1 DNA sequences. Am J Bot 85:1517-1530 doi:Doi 10.2307/2446479

Koopman WJM, Zevenbergen MJ, Van den Berg RG (2001) Species relationships in *Lactuca* SL (Lactuceae, Asteraceae) inferred from AFLP fingerprints. Am J Bot 88:1881-1887 doi:Doi 10.2307/3558364

Kristkova E, Dolezalova I, Lebeda A, Vinter V, Novotná A (2008) Description of morphological characters of lettuce (*Lactuca sativa* L.) genetic resources. Hortic Sci 35:113-129

Kumari A, Baskaran P, Van Staden J (2016) *In vitro* propagation and antibacterial activity in *Cotyledon orbiculata*: a valuable medicinal plant Plant Cell, Tissue and Organ Culture (PCTOC) 124:97-104

Lebeda A (1998) Biodiversity of the interactions between germplasms of wild *Lactuca* spp. and related genera and lettuce downy mildew (*Bremia lactucae*). Horticulture Research International, Wellesbourne (UK)

Lebeda A, Astley D World genetic resources of *Lactuca* spp., their taxonomy and biodiversity. In: Eucarpia Leafy Vegetables' 99, Olomouc (Czech Republic), 8-11 Jun 1999, 1999. Palacky University,

Lebeda A, Dolezalova I, Astley D (2004) Representation of wild *Lactuca* spp. (Asteraceae, Lactuceae) in world genebank collections. Genet Resour Crop Evol 51:167-174 doi:10.1023/B:GRES.0000020860.66075.f7

Lebeda A, Dolezalova I, Kristkova E, Kitner M, Petrzelova I, Mieslerova B, Novotna A (2009) Wild *Lactuca* germplasm for lettuce breeding: current status, gaps and challenges. Euphytica 170:15-34 doi:10.1007/s10681-009-9914-7

Lebeda A, Kitner M, Křístková E, Doležalová I, Beharav A (2012) Genetic polymorphism in *Lactuca aculeata* populations and occurrence of natural

putative hybrids between *L. aculeata* and *L. serriola.* Biochem Syst Ecol 42:113-123

Lebeda A, Ryder EJ, Grube B, Dolezalova I, Kristkova E. Singh RJ (2007) Lettuce (Asteraceae; *Lactuca* spp.), Genetic resources, chromosome engineering, and crop improvement: vegetable crops , Vol. 3 Boca Raton (FL)CRC Press(pg. 377-472)

Leck MA, Parker VT, Simpson RL (2008) Seedling ecology and evolution. Cambridge University Press: Cambridge, UK

Lindqvist K (1958) Inheritance of lobed leaf form in *Lactuca.* Hereditas 44:347-377

Malarz J, Stojakowska A, Kisiel W (2002) Sesquiterpene lactones in a hairy root culture of *Cichorium intybus.* Z. Naturforschung C 57:994-997

Michalska K, Stojakowska A, Malarz J, Doležalová I, Lebeda A, Kisiel W (2009) Systematic implications of sesquiterpene lactones in *Lactuca* species. Biochemical Systematics and Ecology 37:174-179

Mizutani T, Liu XJ, Tashiro Y, Miyazaki S, Shimasaki K. 1989. Plant regeneration and cell fusion of protoplasts from lettuce cultivars and related wild species in Japan. Bull. Fac. Agric. Saga. Univ. 67:109–118

Mohebodini M, Mokhtar JJ, Mahboudi F, Alizadeh H (2011) Effects of genotype, explant age and growth regulators on callus induction and direct shoot regeneration of Lettuce (*Lactuca sativa* L.) Aust J Crop Sci 5:92

Moles AT et al. (2009) Global patterns in plant height. Journal of Ecology 97:923-932

Mondini L, Noorani A, Pagnotta MA (2009) Assessing plant genetic diversity by molecular tools. Diversity 1:19-35

Mou B (2008) Lettuce. In: Vegetables I. Springer, pp 75-116

Murthy HN, Kim Y-S, Park S-Y, Paek K-Y (2014) Hypericins: biotechnological production from cell and organ cultures. Applied microbiology and biotechnology 98:9187-9198

Nei M (1987) Molecular evolutionary genetics. Columbia university press

Nishimura K, Miyase T, Ueno A, Noro T, Kuroyanagi M, Fukushima S (1986) Sesquiterpene lactones from *Lactuca laciniata.* Phytochemistry 25:2375-2379

Obembe OO, Bello OA, Aworunse OS, Popoola JO, Akposibruke O, Olukanmi BI, Olayode MN (2017) *In vitro* Multiple Shoots Formation in Wild

Lettuce (*Launaea taraxacifolia*)(Willd.) Amin ex C. Jeffrey. Annual Research & Review in Biology 19:1-8

Oliya BK, Kim MY, Lee S-H (2018) Development of genic-SSR markers and genetic diversity of Indian lettuce (*Lactuca indica* L.) in South Korea. Genes & Genomics:1-9

Paek K-Y, Murthy HN, Zhong J-J (2014) Production of biomass and bioactive compounds using bioreactor technology. Springer,

Park J-H, Shin J-H, Roy SK, Park H-Y (2014) Evaluation of cytotoxicity, total phenolic content and antioxidant innate reveal efficient medications in native *Lactuca indica.* Journal of Agricultural Science 6:135

Paul P, Joshi M, Gurjar D, Shailajan S, Kumaria S (2017) *In vitro* organogenesis and estimation of β-sitosterol in *Dendrobium fimbriatum* Hook.: An orchid of biopharmaceutical importance. S Afr J Bot 113:248-252

Pink D, Carter PJ (1987) Propagation of lettuce (*Lactuca sativa*) breeding material by tissue culture Ann Appl Biol 110:611-616.

Poorter L et al. (2008) Are functional traits good predictors of demographic rates? Evidence from five neotropical forests. Ecology 89:1908-1920

Rathcke B, Lacey EP (1985) Phenological patterns of terrestrial plants. Ann Rev Ecol Syst 16:179-214

Rauscher G, Simko I (2013) Development of genomic SSR markers for fingerprinting lettuce (*Lactuca sativa* L.) cultivars and mapping . BMC plant biology 13:11

Riar DS, Rustgi S, Burke IC, Gill KS, Yenish JP (2010) EST-SSR development from 5 *Lactuca* species and their use in studying genetic diversity among *L. serriola* biotypes. J Hered 102:17-28

Ryder EJ (1999) Lettuce, endive and chicory. Cab International, Wallingford

Akpere AM, Ayisire ER, Abioye OI (2011) Potential of *Launea taraxacifolia* (Willd) Amin Ex. C. Jeffrey for *in vitro* regeneration. Notulae Scientia Biologicae 3:93

Salgotra RK, Gupta BB (2015) Plant genetic resources and traditional knowledge for food security. Springer, Berlin. doi:10.1007/978-981-10-0060-7

Seo MW, Yang DS, Kays SJ, Lee GP, Park KW (2009) Sesquiterpene lactones and bitterness in Korean leaf lettuce cultivars. Hort Science 44:246-249

Sessa RA, Bennett MH, Lewis MJ, Mansfield JW, Beale MH, 2000. Metabolite profiling of sesquiterpene lactones from *Lactuca* species. J Biol Chem.275, 26877–26884

Silva E, Maluf W, Leal N, Gomes L (1999) Inheritance of bolting tendency in lettuce *Lactuca sativa* L. Euphytica 109:1-7

Simko I (2008) Development of EST-SSR markers for the study of population structure in lettuce (*Lactuca sativa* L.). J Hered 100:256-262

Smith JM (1989) Evolutionary genetics. Oxford University Press: London, UK

Sun S, Frelich LE (2011) Flowering phenology and height growth pattern are associated with maximum plant height, relative growth rate and stem tissue mass density in herbaceous grassland species. J Ecol 99:991-1000

Taşkın H, Baktemur G, Kurul M, Büyükalaca S (2013) Use of tissue culture techniques for producing virus-free plant in garlic and their identification through real-time PCR The Scientific World Journal 2013

Thompson, RC (1943) Further studies on interspecific genetic relationships in *Lactuca*. J Agr Res 66: 41–48.

Tilman D, Balzer C, Hill J, Befort BL (2011) Global food demand and the sustainable intensification of agriculture. Proceedings of the National Academy of Sciences 108:20260-20264

Upadhyaya H, Gowda C, Sastry D (2008) Plant genetic resources management: collection, characterization, conservation and utilization. J SAT Agr Res 6:16pp

Van Treuren R, Van der Arend A, Schut J (2013) Distribution of downy mildew (*Bremia lactucae* Regel) resistances in a genebank collection of lettuce and its wild relatives. Plant Genet Res 11:15-25

Varshney RK, Graner A, Sorrells ME (2005) Genic microsatellite markers in plants: features and applications. Trends Biotechnol 23:48-55

Varshney RK, Hoisington DA, Tyagi AK (2006) Advances in cereal genomics and applications in crop breeding. Trends biotechnol 24:490-499

Vu V et al. (2015) Natural variation in gene expression modulates the severity of mutant phenotypes. Cell 162:391-402

Wang J, Li J-l, Li J, Li J-x, Liu S-j, Huang L-q, Gao W-y (2017) Production of active compounds in medicinal plants: from plant tissue culture to

biosynthesis. Chinese Herbal Medicines 9:115-125 doi:https://doi.org/10.1016/S1674-6384(17)60085-6

Wang SY, Chang HN, Lin KT, Lo CP, Yang NS, Shyur LF (2003) Antioxidant properties and phytochemical characteristics of extracts from *Lactuca indica.* J Agric Food Chem 51:1506-1512 doi:10.1021/jf0259415

Wang X-X, Gao X, Jia Z-J (2010) Sesquiterpenoids from *Lactuca tatarica.* Fitoterapia 81:42-44

Wei Z, Zhu SX, Van den Berg RG, Bakker FT, Schranz ME (2017) Phylogenetic relationships within *Lactuca* L. (Asteraceae), including African species, based on chloroplast DNA sequence comparisons. Genet Resour Crop Evol 64:55-71 doi:10.1007/s10722-015-0332-5

Witsenboer H, Michelmore RW, Vogel J (1997) Identification, genetic localization, and allelic diversity of selectively amplified microsatellite polymorphic loci in lettuce and wild relatives (*Lactuca* spp.). Genome 40:923-936

Zhang J,Yao JT, Sun ZM, Fu G,Galanin DA, Nagasato C, Motomura T, Hu ZM, Duan DL (2017) Effect of domestication on the genetic diversity and structure of *Saccharina japonica* populations in China. Sci Rep 7:42158

Zhu N, Wang M, Wei G-J, Lin J-K, Yang CS, Ho C-T (2001) Identification of reaction products of (−)-epigallocatechin,(−)-epigallocatechin gallate and pyrogallol with 2, 2-diphenyl-1-picrylhydrazyl radical. Food Chem 73:345-349

Zidorn C (2008) Sesquiterpene lactones and their precursors as chemosystematic markers in the tribe Cichorieae of the Asteraceae. Phytochemistry 69:2270-2296

Chapter I. Development of genic-SSR markers and genetic diversity of Indian lettuce (*Lactuca indica* L.) in South Korea

Abstract

Indian lettuce (*Lactuca indica* L.) is an undomesticated wild plant with high economic potential. We know little about the plant's genome, such as its DNA markers, making genetic research using this plant difficult. In this study, 100 genic simple sequence repeat (SSR) primers with a 99–250 bp target amplicon were synthesized from *L. indica* transcriptomic sequences. These primers were examined in eight diverse *L. indica* accessions, and 90 polymorphic SSRs were obtained. Twenty-three of the ninety polymorphic SSRs were used to investigate transferability to another two *Lactuca* species, *Lactuca serriola,* and *Lactuca sativa*. Genetic diversity was investigated in 77 *Lactuca* accessions, including 73 *L. indica* collected from across South Korea, two *L. serriola*, and two *L. sativa*. Our genic-SSR markers were highly polymorphic with a mean polymorphic information content of 0.61 and, on average, 10.83 alleles per locus. The average expected heterozygosity (0.76) was higher than the observed heterozygosity. An analysis of molecular variance revealed that most of the total variance in our

population is attributable to genetic variation among accessions, rather than among provinces and species. STRUCTURE, unweighted neighbor-joining phylogenetic trees, and principal coordinate analyses resulted in three clusters, where northern and central-southern *L. indica* accessions were grouped into two clusters with some admixture. The *L. serriola* and *L. sativa* accessions did not produce a separate cluster due to a small sample size. These results show our SSR markers will be useful in germplasm assessment and genetic studies of *L. indica* and other *Lactuca* species.

Keywords: heterozygosity; *Lactuca indica*; lettuce breeding; genetic diversity; genic-SSR marker; transferability.

Introduction

Understanding the pattern of wild species' genetic diversity is fundamental for our understanding of conservation and evolutionary processes, and is a prerequisite for the efficient use of undomesticated wild resources in new-crop improvement. There are many wild species that are under-researched, despite their economic value and the importance of their traditional uses through the centuries. Recently, interest in these natural resources has increased because of the necessity to broaden genetic variation in cultivated species. New approaches to the study of genetic variation in both wild species and cultivated varieties, mediated by molecular marker information, are promising avenues to exploit these wild genetic resources.

Indian lettuce (*Lactuca indica* L., 2n=2x=18) is a lactiferous annual wild herb in the *Lactuca* genus of the Asteraceae family that also includes cultivated lettuce (*Lactuca sativa*) and its wild relative, *Lactuca serriola* (El-Esawi and Sammour 2014; Wei et al. 2017). This plant originated in Africa (Jeffrey 1966) and is ubiquitous in diverse habitats of South, East, and Southeast Asian countries (Michalska et al. 2009). In Korea, this wild plant grows in wasteland and rural places along roads, highways, and stream banks. The plant is diverse in morphology with heights ranging from 0.4 to 2 m. The leaf of this plant has been

used in salads and soups (Facciola, 1990). Korean people also use this plant as a spring vegetable for the traditional fermented food ‘Kimchi’. Moreover, the whole plant is rich in a milky sap that flows freely from broken or cut tissues. This milky sap is commonly called latex, an important source of sesquiterpene lactone, which has been used in medicine due to its antioxidant, anti-inflammatory, anti-bacterial, digestive, diuretic, necrotic, and sedative properties (Bown 1995; Ha et al. 2017; Kim et al. 2007; Seo et al. 2009; Wang et al. 2003).

Genetic relationships have been studied in diverse wild *Lactuca* species using molecular markers such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) and internal transcribed spacers (ITS-1) DNA sequences (El-Esawi 2015; Hill et al. 1996; Kesseli et al. 1991; Koopman et al. 1998; Lebeda et al. 2009; Witsenboer et al. 1997). However, these studies have generally compared a small number of *L. indica* accessions with wild relatives of cultivated lettuce. Moreover, SSR markers derived from cultivated lettuce (*L. sativa*) have only been applied to other *Lactuca spp.* and few attempts have been made to develop SSR markers from wild *Lactuca* species.

Recently, transcriptomic sequences were analyzed in Korean *L. indica* flowering time leaves to identify the coding sequences that are involved in the sesquiterpene lactone biosynthesis pathway (Ha et al. 2017). The aim of the

present study was to develop genic-SSR markers based on the transcribed sequences from *L. indica* leaf tissue (Ha et al. 2017) and to investigate the transferability of SSR markers to related *Lactuca* species. Using the SSR markers that we developed, we examined genetic diversity in *L. indica* accessions collected from South Korea.

Materials and methods

Plant materials and DNA extraction

Seventy-three *L. indica* accessions that cover 42 diverse collection sites in South Korea were used in this study (Fig. I-S1, Table I-S1). For 54 of these accessions, live whole plants were sampled from the field in June 2015, and then transplanted at the Seoul National University experimental farm, Suwon, the Republic of Korea (N 37° 16' 12.094", E 126° 59' 20.756"), for seed harvest. Based on the density of *L. indica* plants in natural habitats, one to three plants per site were collected in the range of 1.5 to 40 m. Seeds from the remaining 19 *L. indica* accessions were obtained from the Wild Plant Seed Bank of Korea (www.seedbank.re.kr) and Korean National Arboretum (http://www.forest.go.kr). For SSR marker analysis, the seeds from each accession were sown in a greenhouse on April 29, 2016. Seedlings were transplanted 3 weeks later in the field with a row spacing of 60 cm and planting interval of 30 cm. In addition, two accessions of *L. serriola* and two types of cultivated lettuce, Crisp/Iceberg (*L. sativa* var. *capatita*) and Romaine/Cos (*L. sativa* var. *longifolia*), were used for the marker transferability test and genetic relationship analysis in *Lactuca spp*. Genomic DNA was extracted from healthy young leaves using a GeneAll

Exgene™ Plant SV kit (GeneAll Biotechnology Co., Seoul, Korea) following the manufacturer's protocol. The intactness and quality of extracted DNA were examined on 0.8% agarose gel. The concentration of each DNA sample was measured on an ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and diluted to a working concentration of 30 ng/µl with Tris-EDTA buffer (pH 8.0).

Table I-S1 Coordinates of collection sites of Indian lettuce (*Lactuca indica*) accessions sampled in South Korea

Province	Collection site code	Longitude(decimal degree)	Latitude (decimal degree)	Number of accessions per collection site
Chungbuk	Chbuk1	127.5995028	36.7752889	2
	Chbuk2	127.7282111	36.5240528	1
	Chbuk3	127.8227	36.2904333	3
	Chbuk4	127.5725728	36.4920811	2
	Chbuk5	127.7	36.8	1
Chungnam	Chunam1	126.812121	36.91405	1
	Chunam2	126.9813389	36.5600583	2
	Chunam3	126.9375528	36.3872944	1
	Chunam4	126.5002750	36.1458028	2
	Chunam5	126.326708	36.685071	1
	Chunam6	126.393544	36.714847	2
Gangwon	Gawon1	127.8483111	37.6664083	1
	Gawon2	127.2303917	38.1879833	3
	Gawon3	127.98493	37.491757	2
	Gawon4	127.735029	38.2011737	2
	Gawon5	127.663967	38.314887	2
Gyeongnam	Gynam1	128.0183111	34.8977861	1
	Gynam2	128.7778167	35.5108917	2
	Gynam3	127.9017972	34.6815722	1
	Gynam4	127.992407	34.723708	1
Gyeongbuk	Gybuk1	129.0632167	35.6162222	2
	Gybuk2	129.0590222	35.7031861	1
	Gybuk3	129.3794694	36.3495056	1
	Gybyk4	128.68065	36.4399861	1
	Gybuk5	128.68065	36.5848806	3
	Gybuk6	128.5274806	36.8501083	2
Gyeonggi	Gyggi1	126.6804111	37.7948	1
	Gyggi2	127.0663306	38.0226139	3
	Gyggi3	127.1659460	37.754571	2
	Gyggi4	127.165946	37.754571	2
	Gyggi5	129.0102656	36.1202472	2
Jeonbuk	Jebuk1	126.6750639	35.4704333	1
	Jebuk2	127.4205028	35.4392194	2
	Jebuk3	127.3269417	35.7126833	1
	Jebuk4	127.3120889	35.5591667	3
Jeonnam	Jenam1	126.4946278	35.0895194	3
	Jenam2	126.511375	34.7513278	1
	Jenam3	126.2931222	34.5259944	2
	Jenam4	127.3062833	34.838425	1
	Jenam5	127.6859056	34.9358444	1
	Jenam6	127.4243083	35.2608444	3
	Jenam7	126.6374663	34.365353	2
Total	42			73

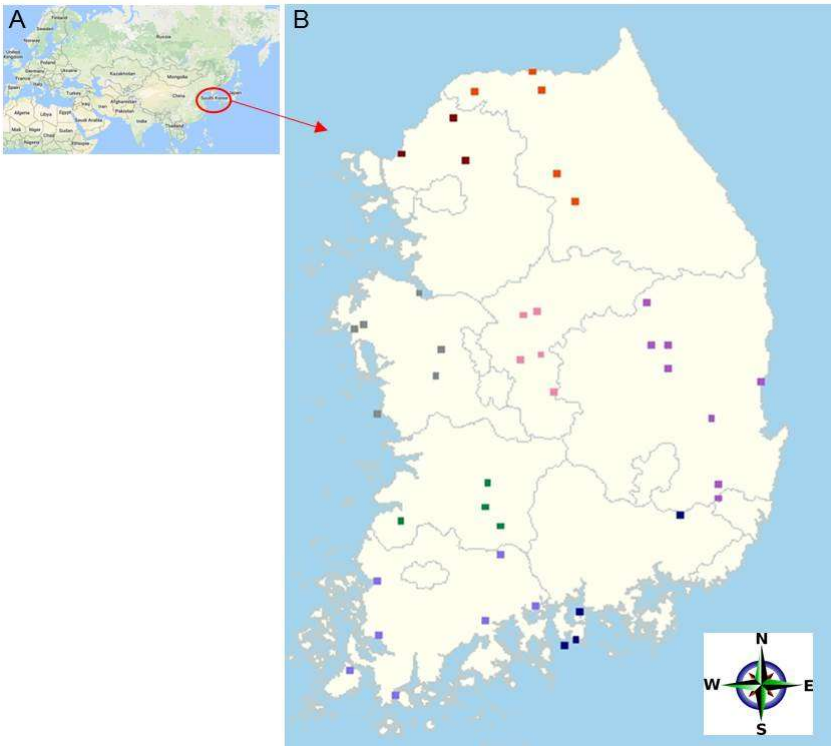

Figure I-S1 Map of South Korea showing 42 collection sites for *L. indica* used in this study. Square spots with the same color represent the collection sites belonging to the same provinces. Map source: the map of Korea is produced in R software using the spatial data provided by http://gadm.org

SSR marker development

From the *L. indica* transcriptomic data reported in the previous study (Ha et al. 2017), nucleotide sequences containing di-, tri-, tetra-, penta-, and hexa-nucleotide repeat motifs with repeat length ≥30 bp were selected to design SSR primers using PRIMER 3 software (Untergasser et al. 2012), with the optimum conditions set at a melting temperature of 50–60°C and a product size range of 99–250 bp. PCR amplification was performed on a final volume of 20 µl containing 30 ng of genomic DNA, 1× PCR buffer, 160 µM of each of the dNTPs, 0.8 U of *Taq* DNA polymerase (VIVAGEN Co., Dajeon, Korea), and 0.5 µM each primer. The thermal cycling condition was carried out in a T100™ Thermal Cycler (BIO-RAD, Hercules, CA, USA) programmed at 94°C for 5 min followed by 34 cycles of denaturing at 94°C for 45 s, annealing at 50–60°C for 45 s, extension at 72°C for 30 s, and then a final extension at 72°C for 6 min. To test the utility of the primers, PCR products were detected on 2% agarose gels. SSR genotyping was performed using a Fragment Analyzer Automated CE System (Advanced Analytical Technologies, Ames, IA, USA) using a DNF-905 dsDNA reagent kit (Advanced Analytical Technologies). Allele sizes and number of alleles per locus (N) were called using PROSize software version 2.0 (Advanced Analytical Technologies).

Genetic diversity analysis

Twenty-three SSR markers with allelic polymorphisms and the same annealing temperature (55°C) were selected for further genetic diversity analysis in 77 accessions of *Lactuca* spp. comprising 73 *L. indica*, two *L. serriola*, and two *L. sativa*. For each SSR marker, the polymorphic information content (PIC) was calculated using $PIC = 1 - \Sigma Pi^2$, where Pi^2 referred to the sum of the i^{th} allelic frequency of each microsatellite locus to all accessions. Genetic diversity parameters were calculated for each marker and each population using Popgene version 1.32 (Yeh et al. 1999), including N, effective number of alleles (Ne), Shannon's information index (I), observed heterozygosity (Ho), expected heterozygosity (He), Nei's expected heterozygosity (Nei), and Nei's genetic distance. The resulting Nei's genetic distance matrix was used to construct an unweighted neighbor-joining (NJ) phylogenetic tree with 1000 bootstrap replications, which was drawn using Mega 6.0 program (Tamura et al. 2013). Similarly, Nei's genetic distance matrix was used for Principal Coordinate Analysis (PCoA) using GenAlEx 6.5 (Peakall and Smouse 2012) to show the pattern of genetic differentiation. To assess the percentage distribution of genetic variation and fixation index (F_{ST}) among and within provinces and species, a

hierarchical analysis of molecular variance (AMOVA) was performed using GenAlEx 6.5 software (Peakall and Smouse 2012).

Population structure analysis

A Bayesian clustering method was implemented to deduce population structure using STRUCTURE 2.3.4 software (Pritchard et al. 2000). The admixture model with correlated allele frequencies was used as suggested in the manual. The estimated number of populations (K) was set from 1 to 15, with a 100,000-step burn-in and 500,000 Markov Chain Monte Carlo (MCMC) iterations. This process was repeated 20 times for each K. The probability values were averaged across runs for each cluster. This procedure clusters individuals in populations and estimates the proportion of membership in each population for each individual (Pritchard et al. 2000). The optimal K value was determined using log likelihood (LnP (D)) based on the rate of change in LnP (D) between successive Ks and the ΔK method (Evanno et al. 2005), using the web-based software STRUCTURE HARVESTER version 0.6.92 (Earl and Vonholdt 2012).

Results

SSR marker development in *L. indica*

One hundred SSR primer pairs designed from the transcribed sequences in the *L. indica* leaves were screened for specific PCR amplification against eight *L. indica* accessions. Three SSR primers failed PCR amplification. The remaining 97 SSR primer pairs, consisting of 61 pairs with di-, 25 with tri-, one with penta-, and 11 with hexa-nucleotide repeat motifs, were effective and reliable for the amplification of target amplicons. Among them, 90 primers produced polymorphic DNA bands among the eight accessions. For each of the SSR markers in *L. indica*, the name, sequence of the forward and reverse primers, repeat type, expected and observed size of the PCR products and annealing temperature are listed in Supplementary Table (Table I-S1).

Table I-S2 Forward and reverse primer sequences, the repeat type, annealing temperature, expected and observed sizes of the PCR products for 100 SSR loci examined in 8 diverse *L. indica* accessions

SSR locus	SSR motif	Forward primer (5'-3')	Reverse primer (5'-3')	Target size (bp)	Tm (ºC)	PCR amplification	Allele size (bp)
Li-1	(GA)22	TTTACCTCCCACCACACACA	CCGGCGATTCCATTATTGAT	245	55	+	232-278
Li-2	(TAT)15	GCATTCTCGACATTTCCG	GGAGAGGGAGGATCATAGCC	106	55	+	96-118
Li-3	(GA)21	CCACTGAAAGCTCGACTGGT	TGATTGTCGCCACCAAAATA	144	55	+	139-178
Li-4	(AGA)16	GAGATGGCGATTTTGGACAT	TGCTGTCTGTCCATCCTCAG	219	55	+	199-215
Li-5	(CT)23	TTCTTTCAAACGAATCCAGACA	TCATCAGTTCCGGCATGTTA	190	52	+	176-193
Li-6	(AG)20	GAGTCCTTTTACATATTTCCATTGC	ACAGGAATCTGGGCTTGATG	221	52	+	199-219
Li-7	(CT)21	GACATAACCAGGTGGTGGGT	TCCCTCATACAAACCCCACT	248	52	+	236-245
Li-8	(CT)20	GACATAACCAGGTGGTGGGT	TCCCTCATACAAACCCCACT	246	55	+	234-247
Li-9	(AG)20	GGCGGTCGGTAAAGGTGTA	CACCACAGATTCACCACGAC	190	55	+	194-206
Li-10	(GA)21	CCAGCCGATCAATTTCTCAT	CATGTTCGATGCCAACTACG	130	52	+	194-206
Li-11	(CT)21	TGGGGGTTGAAAGAGATGAG	CCGACTATTTTAAATCAAGAAATCC	151	52	+	120-149
Li-12	(GA)20	ACTCTCAATTTCGCCACCAC	ATCGGTTCTTGTTTTGGCTG	149	55	+	278-308
Li-13	(CT)26	GTAAGCCGTTCCGATCACTC	CACCCCATTTGTCCTTCTGT	126	52	+	92-117
Li-14	(GA)20	GTTGGGACCCTGAACTTGAA	TTGGCATACAATCTAGAACTTTGT	203	52	+	205-221
Li-15	(ATC)14	GAACATTGTCACAAGAGGGTGA	CATCATTGCCTTGTAGGGGT	245	55	+	226-242
Li-16	(GA)23	CTGCAATACATGCACACACG	CCGCGTCCCAGATATAGGTA	106	52	+	111-142
Li-17	(TCA)16	AGCTTGCTCAACTTGCCATT	CCATTTGAGGAGAACCCTGA	230	52	+	67-79
Li-18	(TCA)18	AGCTTGCTCAACTTGCCATT	CCATTTGAGGAGAACCCTGA	236	52	+	68-74
Li-19	(TCA)17	AGCTTGCTCAACTTGCCATT	CCATTTGAGGAGAACCCTGA	233	52	+	69-76
Li-20	(TCA)15	AGCTTGCTCAACTTGCCATT	CCATTTGAGGAGAACCCTGA	227	52	+	70-76
Li-21	(GA)24	CCTGCAAAAGAATCATTGAAAA	CAACTGAAATGGCTTGCTCA	99	52	+	75-88
Li-22	(AG)23	CGACGTTTGAAGCTGAATGA	TATACACCTCCCTCCCACCC	214	52	+	68-93
Li-23	(AG)22	CGACGTTTGAAGCTGAATGA	TATACACCTCCCTCCCACCC	212	55	+	72-79
Li-24	(AG)21	CGACGTTTGAAGCTGAATGA	TATACACCTCCCTCCCACCC	210	55	+	67-77
Li-25	(AG)20	CGACGTTTGAAGCTGAATGA	TATACACCTCCCTCCCACCC	208	55	+	63-78
Li-26	(GA)21	GAGTGAAACTGGCGTAAGGC	CTCTCTCTCCGCCGTCAA	154	52	+	125-130
Li-27	(CT)20	CGGAGCAGACTCCTGATGA	TGCAAGTGACCACTCTTTCTG	148	55	+	128-165
Li-28	(GAA)14	TTCAATCGGAAAGAAAACGG	CACAGGATCTTGCCATGAGA	221	55	+	300-407
Li-29	(TTC)15	GCGCCCATCAAGATTTGT	TGCAATTGACCACCAGAAAA	216	53	+	333-365
Li-30	(AG)21	CGACGTTTGAAGCTGAATGA	TATACACCTCCCTCCCACCC	210	52	+	354-376
Li-31	(AC)19	GGCTTTAATGTCTACCCATTCC	TGAGCAACGGGGAGTAAAAC	150	52	+	354-365
Li-32	(AG)15	ACAATGTGGCACCAAGCATA	TGGATATTTACATTCACCCACAA	106	52	+	140-298
Li-33	(AG)15	AACGTCAGATTGTCACGCAG	ACGCCATCATCAATCAATCA	207	55	+	203-227
Li-34	(AG)16	AGTGAAGCCCGAAAGTGAGA	TCACACACCAACATATCCCAA	173	55	+	199-273

Li-35	(AG)17	CTGTTGGCTTCCTGGCTCT	ACGGGAGATTGAGGAGATTG	121	55	+	mono (123)
Li-36	(AG)17	TGAATCAATCGCCACCATTA	AGACGTTTTGTTCGTTTGGG	158	55	+	85-93
Li-37	(AG)17	TGAAAGGGTTGTTGTGGTGA	CTCCTCCGAGCACAACCAG	248	55	+	197-219
Li-38	(AG)18	TTGAGTTCCACCACCTGACA	CCAGACGCCTCTCTGTCTCT	121	55	+	172-190
Li-39	(AG)18	AACGTCAGATTGTCACGCAG	ACGCCATCATCAATCAATCA	213	55	+	200-217
Li-40	(AG)19	CGACGTTTGAAGCTGAATGA	TATACACCTCCCTCCCACCC	206	53	+	73-78
Li-41	(AT)15	TTTTCGCTTTCTTCATGCCT	AGACCCGAGTTCCACCAGAT	113	53	+	mono (104)
Li-42	(CT)15	ATGCTTTCGACAAAGCGTCT	CGGGTTAGCTTATTTTGTCAGC	144	53	+	233-243
Li-43	(CT)16	TTTAAAGCTTCTTCCTATTTCCC	AGACCACGAACGAGAGAAGG	123	55	+	78-83
Li-44	(CT)16	AACCCACAAAACAAGAACGC	AAACAACCCATGCCTGTTTC	241	55	+	163-409
Li-45	(CT)17	GTAAGCCGTTCCGATCACTC	CACCCCATTTGTCCTTCTGT	108	55	+	230-250
Li-46	(CT)17	CCAAATTACAACCCGACCTG	GAAGAAGGTGGTGGGTACGA	202	52	+	194-231
Li-47	(CT)18	TCGTCATCTCCTTTTCTCATTTC	CACACGCGTTGTCTATGGAG	129	52	+	136-140
Li-48	(CT)19	TCCATAACTAAGGGTCATTTGC	GTGGGTTGACTCGTTGACCT	106	52	+	89-126
Li-49	(CT)19	GACATAACCAGGTGGTGGGT	TCCCTCATACAAACCCCACT	244	55	+	153-202
Li-50	(GA)15	TGAGGAGGAGTTTCACAGCA	TTGCTGCACTCCACATTACC	141	53	+	100-111
Li-51	(GA)15	TACGTCCACGACCAATACGA	TTCGTCAACGTTTTTAGGGC	177	55	+	124-149
Li-52	(GA)15	AACCGATTTGTGCAAAGGAG	TGCAATTTGCCGCTTAGAAT	233	55	+	281-306
Li-53	(GA)16	AAAACAACCACCATCGGAAA	ATCGGTTCTTGTTTTGGCTG	182	55	+	91-225
Li-54	(GA)16	TGAGTGAAGCGATGGTCAAG	CAAGGCCAATAATTTCAGGG	222	53	+	185-193
Li-55	(GA)17	GCAGAGATTTTAATAGAAATTGTAACG	TTTGAGCCACTCTGTTGTCG	109	55	+	98-125
Li-56	(GA)17	TGAGTGAAGCGATGGTCAAG	CAAGGCCAATAATTTCAGGG	224	52	+	188-193
Li-57	(GA)18	AATGGTGGATGCGGTTATGT	TCACTGTAATCGCCGACACT	162	52	+	mono (117)
Li-58	(GA)18	ATGGTAATACCCATGGCTGG	TAAAGAACATCCCCTCCCCT	209	55	+	192-220
Li-59	(GA)18	TGAGTGAAGCGATGGTCAAG	CAAGGCCAATAATTTCAGGG	226	55	+	137-157
Li-60	(GA)19	TGCGTCAAACTCTTCTACCTCTT	GGTTACGGTTACTGCTCCGA	180	55	+	222-235
Li-61	(GA)19	GTTGTTGAATCGGAATCGCT	TCCAGTTACGCCACAATCAA	103	55	+	89-113
Li-62	(GA)19	TGAGTGAAGCGATGGTCAAG	CAAGGCCAATAATTTCAGGG	228	55	+	222-235
Li-63	(TC)15	CGTCGTCGATCTATCCGTTA	GAGCTGCAGTTGGGTCTTTC	248	55	+	247-259
Li-64	(TC)16	GGATTCGGAGAAGGTGTGAA	GAGAGAGAAAGGGTCATGCAG	131	53	+	mono (113)
Li-65	(TC)17	GTCGGGTATGAGGATGAGGA	AGAAATTGATGGATGGCGAG	169	55	+	62-75
Li-66	(TC)18	TCTAACCTGCTTCTCGAACG	CAATCGCTTCTCTTTCGCTC	200	53	+	58-78
Li-67	(TC)19	GTCGACAGCTCTCCGATTGT	CCATTTGAAGGAAGGAAGCA	250	53	+	67-78
Li-68	(TC)19	TGAGCCCTATTCAACGTTCC	TATGTAACCGCGACGTACCC	191	55	+	65-77
Li-69	(TG)15	GGGGATACATATGTTGCTACCG	TTGTTCCTTGAAATGTCACCA	185	55	+	115-120
Li-70	(TG)15	TCCATGGAAACGACAAGTGA	CCGTCTGCCTTTCATTCATT	247	53	+	129-169
Li-71	(TGA)11	AATGATGATGATGGCAATGG	CATGTCGACAAGAAAGTCCA	115	52	+	95-112
Li-72	(TTTTG)6	CTCCAGCAGCTTCCCTATTG	TTGTGATGCAGTTCAACAATCA	130	55	+	100-300
Li-73	(AAGGTC)5	TGGCATTGGCTACACAGAAG	ACGGAATTCACATTTCTGCC	231	55	+	Mono 233)
Li-74	(ACATGA)6	ACAAGGGCTCTGTTGACCAT	CTGTCCAACGTGAACCAATG	233	55	+	200-244
Li-75	(ATCAAA)5	TACTTCACTTGCACCGAACG	GCTTCTTTACTGAGGCCACG	226	55	+	mono (228)
Li-76	(CATTCC)5	CAGCCATTGGATCATTCTGA	AATGGCTATGATGAACCCCA	217	55	+	225-216

Li-77	(GAAATT)5	TGGAAATCCAATTCTGCCTC	CAAAGTCGAAGCTCACTCCC	162	55	+	161-166
Li-78	(GCGGTG)5	TGAAAAATTACGCCGAGGAC	TCATTAGCCTCCACTCTCCG	243	55	+	225-240
Li-79	(GCTGAT)5	GAAACGGAAACGGAAACAGA	CCTCTGGTTTCTCCCACAGA	221	55	-	-
Li-80	(GGAACC)5	TCTTCAACTCCGACAAACCC	TTCCAGTTCCAGTCCCAGTC	99	55	+	mono (92)
Li-81	(GGAACC)6	TCTTCAACTCCGACAAACCC	TTCCAGTTCCAGTCCCAGTC	105	55	+	88-106
Li-82	(TCTTAT)6	AATTGGGATCATTCCTCACG	CAATAATTGGGGGAACCTGA	240	55	-	-
Li-83	(TCTTTC)6	TGTTTTCTTTACCTTTGAGATTCTTC	CCCCTGTGTTGGATTTGTCT	202	55	+	193-208
Li-84	(TTGGGT)5	AAAACCCACCTCCACACAAA	ATTGAAGGTGCCGGTTGTAG	217	55	+	260-268
Li-85	(TTTGAC)5	GTTCCTCTGCATCGGATTGT	CCCGAGATCTTTCCATTCAA	176	55	+	225-243
Li-86	(AGA)10	CCCCAAAAGTATTGGAAAAACA	AACCCTACACTCCCTCCACC	208	53	-	-
Li-87	(ATA)10	ATTATTCTCAGGGAGGCGCT	CCCAATTAAAAATCCAAAACCA	124	53	+	128-133
Li-88	(ATA)10	AATGGGAGGGAATCGTGG	CAACAAATCCATGCAACAGG	210	55	+	204-216
Li-89	(ATA)11	AATGGGAGGGAATCGTGG	CAACAAATCCATGCAACAGG	213	55	+	207-216
Li-90	(ATA)13	GTAACTGTTGCTACAAAAATCAAAAT	TAAGAAACGCAAGCTCGGAT	160	55	+	136-139
Li-91	(ATC)10	ATGGATGGACATGGACAGAC	AAGAGAAGACGAGCGATCCA	188	55	+	167-192
Li-92	(ATC)12	GAACATTGTCACAAGAGGGTGA	GGTCAAAGGGGTTGATGATG	229	55	+	200-225
Li-93	(ATT)10	GGAGATGGAGGGATGTTGAA	TCCACTGAAAATGTGACCCA	197	55	+	221-240
Li-94	(ATT)11	GGAGATGGAGGGATGTTGAA	TCCACTGAAAATGTGACCCA	200	55	+	195-211
Li-95	(CCT)11	GCTCAAGTCAACGGTCTTCC	GTTGGGCGTAGGAAGTTTGA	216	55	+	207-214
Li-96	(CTG)11	AATTGGCTCCTCCCAAGACT	ATCATCGGCTGTTTCCACTC	178	55	+	425-485
Li-97	(GAA)10	TTCAATCGGAAAGAAAACGG	CACAGGATCTTGCCATGAGA	209	55	+	379-400
Li-98	(TCA)12	AAAATCCAAAAACCCTCGCT	AACGCGGTGAAGCTGTATCT	187	55	+	183-206
Li-99	(TCA)12	CACGGGATTTTCTATTTCATGC	CCAAATGTGCAATTTTGTGC	239	55	+	232-246
Li-100	(TCT)11	CTCAATCGCAAATCTGACCC	AAGCTCTTCGCCAACCAGTA	169	55	+	163-195

Twenty-three SSR primers were randomly selected to test transferability to other two *Lactuca* species, *L. serriola* and *L. sativa*. Out of them, twenty primers successfully amplified specific PCR fragments in both of these species (Table I-1), which indicate that the genic-SSR markers developed in this study can be effectively used in genetic diversity studies not only in *L. indica*, but also in *L. Sativa* and *L. serriola*. Using 23 SSR primers, 5 to 23 alleles per locus were generated from 73 *L. indica* accessions with total alleles of 237, ranging from 78 to 489 bp in size. From two accessions of *L. serriola*, twenty-two SSR primers yielded 44 alleles but Li-3 primer failed to amplify the DNA fragment. Two *L. sativa* accessions showed 24 alleles in 20 SSR loci but three loci Li-3, Li-9, and Li-84 did not amplify the PCR product (Table I-1).

Table I-1 Primer sequences, repeat motif, amplicon size, and allele number of 23 polymorphic genic-SSR markers in 77 accessions including 73 *L. indica*, two *L. serriola,* and two *L. sativa*

SSR locus	Primer sequences	SSR motif	Amplified allele size	No. of alleles amplified		
				L. indica	*L. serriola*	*L. sativa*
Li-1	F: TTTACCTCCCACCACACACAR: CCGGCGATTCCATTATTGAT	(GA)22	108–332	23	3	1
Li-3	F: CCACTGAAAGCTCGACTGGTR: TGATTGTCGCCACCAAAATA	(GA)21	107–181	18	0	0
Li-4	F: GAGATGGCGATTTTGGACATR: TGCTGTCTGTCCATCCTCAG	(CT)23	183–261	12	3	2
Li-6	F: GAGTCCTTTTACATATTTCCATTGCR: ACAGGAATCTGGGCTTGATG	(AG)20	193–240	12	3	2
Li-9	F: GGCGGTCGGTAAAGGTGTAR: CACCACAGATTCACCACGAC	(AG)20	107–220	17	2	0
Li-10	F: CCAGCCGATCAATTTCTCAT: CATGTTCGATGCCAACTACG	(GA)21	106–124	8	3	1
Li-15	F: GAACATTGTCACAAGAGGGTGAR: CATCATTGCCTTGTAGGGGT	(ATC)14	224–277	9	3	1
Li-27	F: CGGAGCAGACTCCTGATGAR: TGCAAGTGACCACTCTTTCTG	(CT)20	123–228	13	2	1
Li-28	F: TTCAATCGGAAAGAAAACGGR: CACAGGATCTTGCCATGAGA	(GAA)14	397–404	10	1	1
Li-33	F: AACGTCAGATTGTCACGCAGR: ACGCCATCATCAATCAATCA	(AG)15	201–289	11	1	1
Li-63	F: CGTCGTCGATCTATCCGTTAR: GAGCTGCAGTTGGGTCTTTC	(TC)15	228–265	7	2	1
Li-77	F: TGGAAATCCAATTCTGCCTCR: CAAAGTCGAAGCTCACTCCC	(GAAATT)5	140–187	7	2	1
Li-81	F: TCTTCAACTCCGACAAACCCR: TTCCAGTTCCAGTCCCAGTC	(GGAACC)6	78–150	9	2	1
Li-84	F: AAAACCCACCTCCACACAAAR: ATTGAAGGTGCCGGTTGTAG	(TTGGGT)5	255–289	10	2	0
Li-90	F: GTAACTGTTGCTACAAAAATCAAAATR: TAAGAAACGCAAGCTCGGAT	(ATA)13	133–174	7	1	2
Li-92	F: GAACATTGTCACAAGAGGGTGAR: GGTCAAAGGGGTTGATGATG	(ATC)12	204–240	10	2	1
Li-94	F: GGAGATGGAGGGATGTTGAAR: TCCACTGAAAATGTGACCCA	(ATT)11	193–211	7	1	2
Li-95	F: GCTCAAGTCAACGGTCTTCCR: GTTGGGCGTAGGAAGTTTGA	(CCT)11	196–222	7	2	1
Li-96	F: AATTGGCTCCTCCCAAGACTR: ATCATCGGCTGTTTCCACTC	(CTG)11	352–498	9	1	1
Li-97	F: TTCAATCGGAAAGAAAACGGR: CACAGGATCTTGCCATGAGA	(GAA)10	385–409	5	1	1
Li-98	F: AAAATCCAAAAACCCTCGCTR: AACGCGGTGAAGCTGTATCT	(TCA)12	170–195	8	2	1
Li-99	F: CACGGGATTTTCTATTTCATGCR: CCAAATGTGCAATTTTGTGC	(TCA)12	237–258	8	1	1
Li-100	F: CTCAATCGCAAATCTGACCCR: AAGCTCTTCGCCAACCAGTA	(TCT)11	144–192	10	4	1
Total				237	44	24

Genetic diversity analysis

Based on the genotyping of the 23 SSR markers, the PIC values ranged from 0.36 (Li-90 and Li-97) to 0.78 (Li-1) with an average of 0.61 (Table I-2). Overall, 18 SSR primers were considered to be highly informative (PIC ≥ 0.5) and five SSR primers were considered to be reasonably informative (0.5 ≤ PIC ≤ 0.35). We analyzed genetic diversity not only in 73 *L. indica* accessions but also in 77 accessions including two *L. serriola* and two *L. sativa* accessions, summarized in Supplementary Table I-S3 and Table I-2, respectively. Two analyses showed similar values for genetic diversity indices. It is likely that *L. indica* has a wide spectrum of SSR alleles while *L. serriola* and *L. sativa* have a low number of alleles due to a small sample size, as shown in Table I-1. For the whole accessions including three *Lactuca* species, the Ne was in the range of 2.43–10.38, averaging 4.97 over all loci (Table I-2). The average He was 0.76, ranging from 0.59 in Li-94 to 0.91 in Li-1 and Li-3. The average Ho was 0.39, with the lowest value in Li-81 (0.12) and the highest value in Li-4 (0.76). Nei ranged from 0.59 (Li-94) to 0.90 (Li-1 and Li-3) with an average of 0.76. The Hardy–Weinberg equilibrium (HWE) exact test for all populations revealed that all loci significantly deviated from HWE corrected for multiple comparisons ($p < 0.000001$), displaying a level of heterozygosity lower than expected.

Table I-2 Genetic diversity indices of 23 SSR loci in 77 accessions including 73 *L. indica*, two *L. serriola*, and two *L. sativa*

SSR locus	PIC	N	Ne	I	Ho	He	Nei	PHWE*
Li-1	0.78	25	10.02	2.73	0.58	0.91	0.90	< 0.000001
Li-3	0.77	18	10.38	2.52	0.67	0.91	0.90	< 0.000001
Li-4	0.60	14	5.67	2.04	0.76	0.83	0.82	< 0.000001
Li-6	0.73	14	6.26	2.15	0.28	0.85	0.84	< 0.000001
Li-9	0.73	18	9.05	2.49	0.68	0.90	0.89	< 0.000001
Li-10	0.50	7	3.78	1.63	0.50	0.74	0.74	< 0.000001
Li-15	0.48	10	4.05	1.68	0.56	0.76	0.75	< 0.000001
Li-27	0.65	14	3.45	1.81	0.19	0.72	0.71	< 0.000001
Li-28	0.75	10	6.67	2.05	0.45	0.86	0.85	< 0.000001
Li-33	0.79	11	5.38	1.95	0.23	0.82	0.81	< 0.000001
Li-63	0.69	7	4.06	1.62	0.18	0.76	0.75	< 0.000001
Li-77	0.48	8	2.50	1.30	0.23	0.60	0.60	< 0.000001
Li-81	0.76	10	5.13	1.84	0.12	0.81	0.80	< 0.000001
Li-84	0.63	10	4.34	1.71	0.32	0.77	0.77	< 0.000001
Li-90	0.36	8	4.29	1.55	0.75	0.77	0.77	< 0.000001
Li-92	0.72	10	5.81	1.95	0.31	0.83	0.83	< 0.000001
Li-94	0.52	7	2.43	1.16	0.16	0.59	0.59	< 0.000001
Li-95	0.46	7	2.62	1.19	0.21	0.62	0.62	< 0.000001
Li-96	0.50	9	2.57	1.27	0.18	0.62	0.61	< 0.000001
Li-97	0.36	6	2.56	1.15	0.35	0.61	0.61	< 0.000001
Li-98	0.52	8	3.10	1.52	0.31	0.68	0.68	< 0.000001
Li-99	0.59	8	3.05	1.38	0.25	0.68	0.67	< 0.000001
Li-100	0.63	10	7.10	2.12	0.61	0.86	0.86	< 0.000001
Mean	0.61	10.83	4.97	1.77	0.39	0.76	0.76	

PIC, polymorphic information content; N, total number of alleles; Ne, effective number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; I, Shannon's information index; Nei, Nei's expected heterozygosity; PHWE, probability in Hardy–Weinberg equilibrium; * indicates that the p value is significant.

Table I-S3 Genetic diversity indices of 23 SSR loci in 73 *L. indica* accessions

SSR locus	PIC	N	Ne	I	Ho	He	Nei	PHWE*
Li-1	0.79	23.00	9.25	2.66	0.60	0.90	0.89	< 0.000001
Li-3	0.77	18.00	10.38	2.52	0.67	0.91	0.90	< 0.000001
Li-4	0.62	12.00	5.21	1.94	0.75	0.81	0.81	< 0.000001
Li-6	0.74	12.00	5.86	2.05	0.28	0.84	0.83	< 0.000001
Li-9	0.74	17.00	8.81	2.48	0.70	0.89	0.89	< 0.000001
Li-10	0.53	8.00	3.62	1.59	0.51	0.73	0.72	< 0.000001
Li-15	0.50	9.00	3.81	1.64	0.57	0.74	0.74	< 0.000001
Li-27	0.67	13.00	3.28	1.74	0.21	0.70	0.69	< 0.000001
Li-28	0.76	10.00	6.27	2.00	0.47	0.85	0.84	< 0.000001
Li-33	0.81	11.00	5.74	1.98	0.24	0.83	0.83	< 0.000001
Li-63	0.70	7.00	3.83	1.54	0.19	0.74	0.74	< 0.000001
Li-77	0.50	7.00	2.37	1.22	0.24	0.58	0.58	< 0.000001
Li-81	0.77	9.00	4.82	1.74	0.13	0.80	0.79	< 0.000001
Li-84	0.64	10.00	4.28	1.69	0.33	0.77	0.77	< 0.000001
Li-90	0.39	7.00	4.20	1.52	0.76	0.77	0.76	< 0.000001
Li-92	0.74	10.00	5.63	1.93	0.33	0.83	0.82	< 0.000001
Li-94	0.55	7.00	2.28	1.09	0.14	0.57	0.56	< 0.000001
Li-95	0.50	7.00	2.51	1.15	0.21	0.61	0.60	< 0.000001
Li-96	0.55	9.00	2.59	1.29	0.19	0.62	0.61	< 0.000001
Li-97	0.39	5.00	2.39	1.04	0.34	0.59	0.58	< 0.000001
Li-98	0.54	8.00	2.88	1.47	0.33	0.66	0.65	< 0.000001
Li-99	0.62	8.00	3.02	1.38	0.26	0.67	0.67	< 0.000001
Li-100	0.66	10.00	7.11	2.12	0.62	0.87	0.86	< 0.000001
Mean	0.63	10.30	4.79	1.73	0.39	0.75	0.75	

PIC, polymorphic information content; N, total number of alleles; Ne, effective number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; I, Shannon's information index; Nei, Nei's expected heterozygosity; PHWE, probability in Hardy–Weinberg equilibrium; * indicates that the p value is significant

Genetic differentiation and structuring

AMOVA was performed against the accessions by province in *L. indica* (Table I-3). We did not conduct AMOVA by species due to unbalanced sample sizes among the three *Lactuca* species, *L. indica*, *L. serriola* and *L. sativa*. In *L. indica*, variation among accessions within provinces (95%) are much higher than variation among provinces (5%). Distribution of genetic variation for the 23 SSR markers of interest revealed that most of the total variance is attributable to genetic variation among individuals. A common measure for the degree of genetic differentiation is the fixation index F_{ST}, first defined by Wright (1978). F_{ST} among provinces in *L. indica* was 0.047. This values was significant at $p < 0.0001$ as indicated by a randomization test (Table I-3). The average estimate of gene flow that was calculated by gene flow (Nm) = $[(1 / F_{ST}) - 1] / 4$ was 5.069 among the provinces.

Table I-3 Analysis of molecular variance (AMOVA) of the 73 *L. indica* accessions by provinces.

Source of variation	Degree of freedom	Sum of square	Mean squares	Estimated variance	Variation (%)	F_{ST}*	P value
Among provinces	7	144.052	20.579	0.426	5	0.047	< 0.001
Within provinces	138	1,149.540	17.157	8.579	95		< 0.001
Total	145	1293.596		9.005	100		< 0.001

* Pairwise $F_{(ST)}$

The SSR genotyping results were used to perform population structure analysis for 77 accessions under an admixture model using the STRUCTURE program. At K=3, the value of ΔK was highest. This indicated the presence of three different clusters in the accessions. Each of the three clusters includes a different number of accessions, with nine accessions in cluster I, 34 accessions in cluster II, and 34 accessions in cluster III (Fig. I-1). The *L. indica* accessions were divided into three clusters (I, II, and III), of which cluster III harbored only 34 *L. indica* accessions. The *L. serriola* and *L. sativa* accessions did not produce a separate cluster and were included in cluster I or II, along with the *L. indica* accessions, probably due to small sample sizes of *L. serriola* and *L. sativa*. Generally, northern (Gangwon and Gyeonggi) and southern (Jeonbuk and Geonnam) *L. indica* accessions were separated into clusters II and III, with an admixture of some accessions.

Using the Nei's genetic distance derived from genotyping data, an unweighted NJ phylogenetic tree was constructed with the 77 *Lactuca* accessions (Fig. I-2). The resultant tree showed three clear groups with a similar phylogenetic pattern to that of the STRUCTURE analysis. Group, I showed an admixture of the accessions from different geographic locations. The accessions from northern provinces (Gwangon and Gyeonggi) were clustered together in group II, consistent with cluster II produced by the STRUCTURE program. Group II included two *L. sativa* accessions (acc. 76 and 77), one *L. serriola*

accession (acc. 75), and 14 *L. indica* accessions. Most of the accessions from the central (Chungbuk and Chungnam) and southern parts (Geonbuk and Geonnam) of the country were clustered in group III. Among the accessions that were identified as belonging to cluster I by the STRUCTURE analysis (Fig. I-1), six *L. indica* accessions (acc. 33, 40, 47, 56, 71, and 73) and one *L. serriola* accession (acc. 74) were grouped into group III. Although *L. serriola* and *L. sativa* were clustered with *L. indica*, they showed long evolutionary branches in the NJ phylogenetic tree (Fig.I-2). The two-dimensional PCoA also showed a perfect correspondence with three clusters formed in STRUCTURE analysis (Fig.I-3).

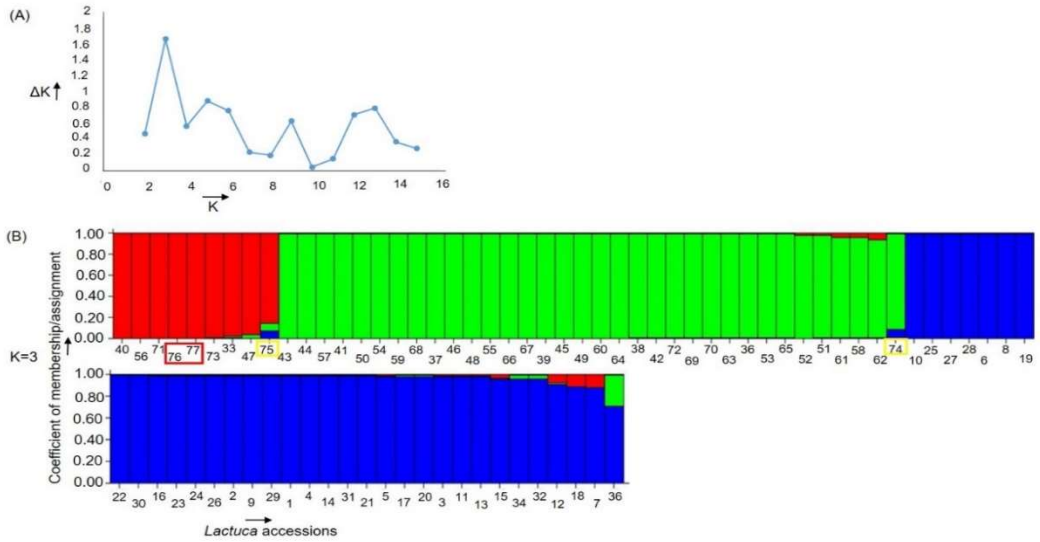

Figure I-1 Population structure of 77 *Lactuca* accessions based on 23 SSR markers. (A) STRUCTURE plot depicting subpopulation following the Evanno method in the STRUCTURE analysis; ΔK reached maximum value when K = 3. (B) Bayesian model estimation of population structure. X-axis represents individual accessions. Y-axis shows coefficient of membership/assignment. Three subpopulation clusters inferred by STRUCTURE are represented by different colors: cluster I, red; cluster II, green; cluster III, blue. Highlighted accessions in red and yellow belong to *L. sativa* and *L. serriola*, respectively.

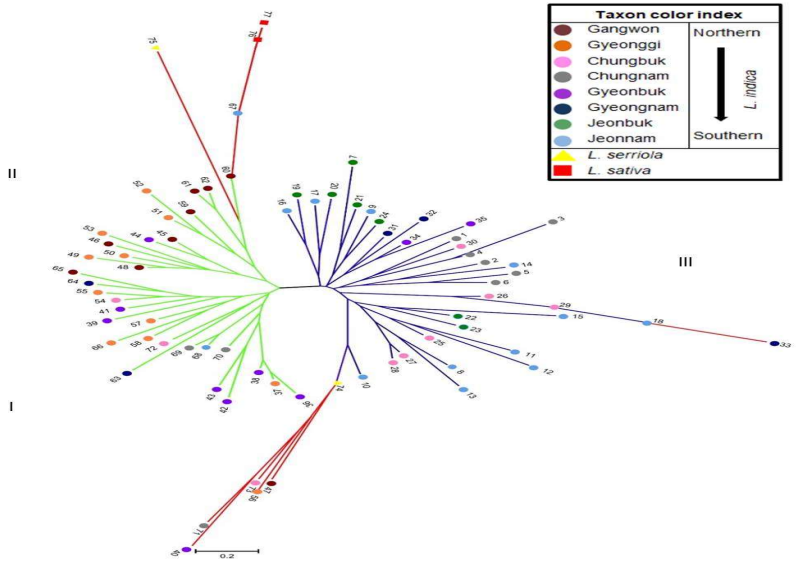

Figure I-2 Neighbor-joining (NJ) phylogenetic tree of the *Lactuca* accessions using Nei's genetic distances based on 23 SSR markers. Acc. 1 to 73, *L. indica*; acc. 74 and 75*, L. serriola*; acc. 76 and 77, *L. sativa*. Taxon color in circle represents *L. indica* belonging to provinces; *L. serriola* and *L. sativa* are indicated by a yellow triangle and a red square, respectively, as shown in the taxon color index. The color of branches indicates accession corresponding to clusters I to III from population structure analysis as in Fig. I-1.

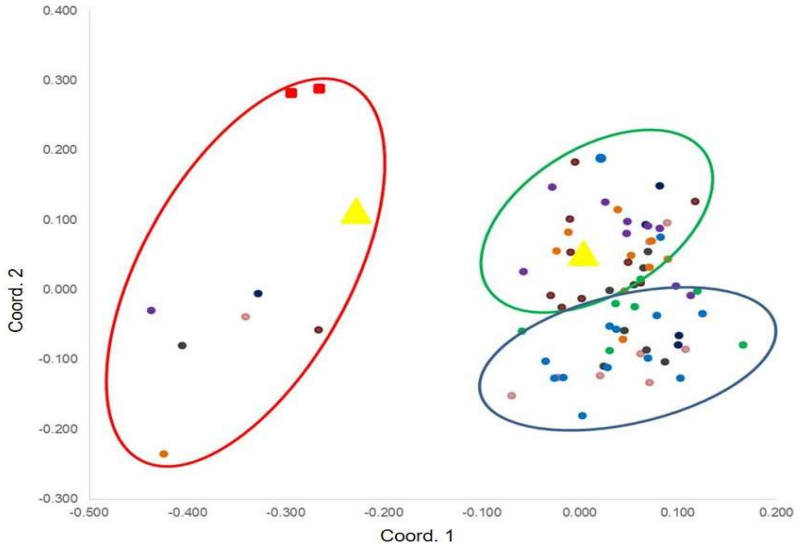

Figure I-3 Principal coordinates analysis (PCoA) bi-plot showing the clustering using Nei's genetic distance among the 77 *Lactuca* accessions based on 23 SSR markers. The percentages of variation explained by the first three axes (1, 2, and 3) are 14.2%, 8.36%, and 6.77%, respectively. Their respective cumulative percentage of variance are 14.27%, 22.63%, and 29.40%. Different circle spots represent L. indica genotypes belonging to eight provinces as indicated in the NJ tree (Fig. I-2). Accessions within different colored elliptical shapes corresponded to the clusters as in STRUCTURE (Fig. I-1) and the NJ tree (Fig. I-2).

Discussion

The present study is the first to develop genic-SSR markers and to demonstrate their use in examining genetic diversity in *L. indica*. One-hundred genic-SSR primers were designed from transcriptomic data of *L. indica* leaves at the flowering stage. These SSR primers showed a PCR success rate of 97% in the *L. indica* accessions (Supplementary Table I-S2). The failure of three primers to amplify genomic DNA of *L. indica* may be attributed to sequence variations in primer-binding sites, the presence of introns or large insertions, or transcript assembly error (Wei et al. 2011; Zhang et al. 2013). The SSR markers developed in *L. indica* could be transferred to other *Lactuca* species, *L. serriola* and *L. sativa*. Similarly, expressed sequence tag (EST)-SSR derived from five *Lactuca* species, *L. sativa*, *L. serriola*, *L. perennis*, *L. virosa*, and *L. saligna*, have been used to study genetic diversity in *L. serriola* and its biotypes *L. sativa* and *L. perennis* (Riar et al. 2010). Van de Wiel et al. (1999) also found SSR transferability of *L. sativa* to other *Lactuca* species. Such cross-amplification of SSR markers among *Lactuca* species may imply a relatively close taxonomic distance among these species. In barley, high levels of transferability of EST-SSR markers to wheat, rye and rice have reported to be accordant with the relative phylogenetic affinities among cereal species (Varshney et al. 2005a; Varshney et al. 2005b).

We used much smaller sample sizes of *L. serriola* and *L. sativa* than *L. indica*. Such unbalanced samples sizes among the species may provide biased information on interspecific phylogenic relationship. In addition, *L. serriola* showed a null allele in one SSR marker (Li-3) and *L. sativa* did in three SSR markers (Li-3, Li-9, and Li-84) (Table I-1). For selectively amplified microsatellite polymorphic loci (SAMPL), not only many new alleles but also null alleles have been observed in wild *Lactuca* species compared with *L. sativa*, indicating SAMPL analysis is more applicable to intraspecific than to interspecific comparisons (Witsenboer et al. 1997). Therefore, genotyping data of our SSR markers are not enough for assessing the robust genetic relationships of *L. indica* with *L. serriola* and *L. sativa*, although genetic clustering and PCoA showed some *L. indica* accessions are closer to wild *L. serriola* than cultivated *L. sativa* (Fig. I-2 and I-3). However, the allelic diversity in the *L. indica* accessions collected across South Korea was effectively determined using the developed genic-SSR markers in this study.

High genetic diversity was found among the 73 *L. indica* accessions, even when the samples were all collected from the same country, South Korea (Table I-2). The variation observed among individuals within provinces rather than between provinces contributed to high genetic diversity in our accessions (Table I-3), likely influenced by the presence of heterozygosity caused by open pollination (Loveless and Hamrick 1984). Previous study described the genetic

differentiation of 67 *Lactuca saligna* accessions that originated in four European countries (Czech Republic, France, Italy, Portugal) and three Middle Eastern countries (Israel, Jordan, Turkey) on the basis of AFLP polymorphisms (Kitner et al. 2008). In their study, the genetic variation within particular populations (countries) was low and most of variation was present among the countries. Those large-scale genetic variations derived from eco-geographical conditions may be ascribed to selection and colonization history (Nybom et al. 2014). However, our data showed that, on a finer geographical scale within a single country, the natural samples of *L. indica* are well-differentiated and each sample can represent a unique combination of genotypes. STRUCTURE, unweighted NJ phylogenetic tree, and PCoA analyses, in general, indicated differentiation of northern and central-southern *L. indica* accessions, with some admixture (Figs. I-1, I- 2, and I-3).

Based on RFLP, SSR and AFLP analyses, some studies showed that *L. sativa* and *L. serriola* are closely clustered and the position of *L. indica* is relatively distant from these two species (El-Esawi and Sammour 2014; Hill et al. 1996; Koopman et al. 1998; Witsenboer et al. 1997). Phylogenetic reconstruction of *Lactuca* species using two chloroplast gene sequences (*ndhF* and *trnL-F*) revealed that *L. indica* belonged to Clade 2 next to the crop clade (Clade 1) comprising cultivated lettuce (*L. sativa*) and several wild lettuces such as *L. serriola* and *Lactuca virosa* (Wei et al. 2017). In the present study, *L. serriola* and

L. sativa did not produce a separate group and some *L. indica* accessions were clustered together with the two species (Figs. I-1, I-2, and I-3). It is likely that wild species *L. indica* constitutes a wide genetic base while *L. serriola* and *L. sativa* show a narrow allelic diversity due to a small sample size. However, these results may indicate the possibility of using *L. indica* as a new genetic resource for lettuce breeding, although further study using a large size of samples is required to determine relatedness of *L. indica* with the two species. Wei et al. (2017) have also suggested a similar idea that *L. indica* carrying high resistance to downy mildew (van Treuren et al. 2013) provide rich genetic resources for the crop lettuce.

In conclusion, the development of highly efficient co-dominant genic-SSR markers in *L. indica*, a species with high industrial, breeding, and commercial potential, enabled us to comprehend genetic diversity and population structure of this species in South Korea, but also to gain such information about other *Lactuca* species. In addition, our results may show the possibility of using *L. indica* as a new genetic resource for the crop lettuce. Thus, our SSR marker information will be very useful in germplasm assessment, biodiversity, and genetic studies of *L. indica* and other *Lactuca* species. In turn, such efforts will no doubt lead to further improvement of the *L. indica* plant as a crop and the introduction of its valuable traits into cultivated lettuce.

References

Bown D (1995) The Royal Horticultural Society encyclopedia of herbs & their uses. Dorling Kindersley Limited, London

Earl DA, Vonholdt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conser Genet Resour 4:359-361. doi:10.1007/s12686-011-9548-7

El-Esawi MA, Sammour R (2014) Karyological and phylogenetic studies in the genus *Lactuca* L. (Asteraceae). Cytologia 79:269-275. doi:10.1508/cytologia.79.269

El-Esawi MA (2015) Molecular genetic markers for assessing the genetic variation and relationships in *Lactuca* germplasm. Annu Re Rev Biol 8:1-13. doi:10.9734/ARRB/2015/20647

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14:2611-2620. doi:10.1111/j.1365-294X.2005.02553.x

Facciola S (1990) Cornucopia: a source book of edible plants. Kampong Publ, Vista, Germany

Ha J, Lee T, Kim MY, Oliya BK, Gwag J-G, Lee Y-H, Lee S-H (2017) Comprehensive transcriptome analysis of *Lactuca indica* L., a traditional medicinal wild plant. Mol Breed 37:112. doi:10.1007/s11032-017-0711-z

Hill M, Witsenboer H, Zabeau M, Vos P, Kesseli R, Michelmore R (1996) PCR-based fingerprinting using AFLPs as a tool for studying genetic relationships in *Lactuca spp*. Theor Appl Genet 93:1202-1210. doi:10.1007/s11032-017-0711-z

Jeffrey C (1966) Notes on the Compositae: I. The Cichorieae in East Tropical Africa. Kew Bulletin 18:427–486

Kesseli R, Ochoa O, Michelmore R (1991) Variation at RFLP loci in *Lactuca* spp. and origin of cultivated lettuce (*Lactuca sativa*). Genome 34:430-436. doi:10.1139/g91-065

Kim KH, Kim YH, Lee KR (2007) Isolation of quinic acid derivatives and flavonoids from the aerial parts of *Lactuca indica* L. and their hepatoprotective activity *in vitro*. Bioorg Med Chem Lett 17:6739-6743. doi:10.1016/j.bmcl.2007.10.046

Kitner M, Lebeda A, Dolezalova I, Maras M, Kristova E, Nevo, E, Pavlicek T, Meglic, V, Beharav, A (2008) AFLP analysis of *Lactuca saligna*

germplasm collections from four European and three Middle Eastern countries. Isr J Plant Sci 56:185-193 doi:10.1560/Ijps.56.3.185

Koopman WJ, Guetta E, van de Wiel CC, Vosman B, van den Berg RG (1998) Phylogenetic relationships among *Lactuca* (Asteraceae) species and related genera based on ITS-1 DNA sequences. Am J Bot 85:1517-1530. doi:10.2307/2446479

Lebeda A, Dolezalova I, Kristkova E, Kitner M, Petrzelova I, Mieslerova B, Novotna A (2009) Wild *Lactuca* germplasm for lettuce breeding: current status, gaps and challenges. Euphytica 170:15-34. doi:10.1007/s10681-009-9914-7

Loveless MD, Hamrick JL (1984) Ecological Determinants of Genetic-Structure in Plant-Populations. Ann Rev of Ecol Syst 15:65-95. doi:10.1146/annurev.es.15.110184.000433

Michalska K, Stojakowska A, Malarz J, Doležalová I, Lebeda A, Kisiel W (2009) Systematic implications of sesquiterpene lactones in *Lactuca* species. Biochem Syst Ecol 37:174-179. doi:10.1016/j.bse.2009.02.001

Nybom H, Weising K, Rotter B (2014) DNA fingerprinting in botany: past, present, future. Investig Genet 5:1. doi:10.1186/2041-2223-5-1

Peakall R, Smouse PE (2012) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288-295. doi:10.1111/j.1471-8286.2005.01155.x

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945-959

Riar DS, Rustgi S, Burke IC, Gill KS, Yenish JP (2010) EST-SSR development from 5 *Lactuca* species and their use in studying genetic diversity among *L. serriola* biotypes. J Hered 102:17-28. doi:10.1093/jhered/esq103

Seo MW, Yang DS, Kays SJ, Lee GP, Park KW (2009) Sesquiterpene lactones and bitterness in Korean leaf lettuce cultivars. Hort Science 44:246-249

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725-2729 doi:10.1093/molbev/mst197

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3-new capabilities and interfaces. Nucleic Acids Res 40:e115-e115. doi:10.1093/nar/gks596

Van de Wiel C, Arens P, Vosman B (1999) Microsatellite retrieval in lettuce (*Lactuca sativa* L.). Genome 42:139-149. doi:10.1139/g98-119

van Treuren R, van der Arend AJM, Schut JW (2013) Distribution of downy mildew (*Bremia lactucae* Regel) resistances in a genebank collection of lettuce and its wild relatives. Plant Genet Resour 11:15-25. doi:10.1017/S1479262111000761

Varshney RK, Graner A, Sorrells ME (2005a) Genic microsatellite markers in plants: features and applications. Trends Biotechnol 23:48-55. doi:10.1016/j.tibtech.2004.11.005

Varshney RK, Sigmund R, Börner A, Korzun V, Stein N, Sorrells ME, Langridge P, Graner A (2005b) Interspecific transferability and comparative mapping of barley EST-SSR markers in wheat, rye and rice. Plant Science 168:195-202. doi:10.1016/j.plantsci.2004.08.001

Wang SY, Chang HN, Lin KT, Lo CP, Yang NS, Shyur LF (2003) Antioxidant properties and phytochemical characteristics of extracts from *Lactuca indica*. J Agric Food Chem 51:1506-1512. doi:10.1021/jf0259415

Wei WL, Qi X, Wang L, Zhang Y,Hua W, Li D, Lv H, Zhang X (2011) Characterization of the sesame (*Sesamum indicum* L.) global transcriptome using Illumina paired-end sequencing and development of EST-SSR markers. BMC Genomics 12:451. doi:10.1186/1471-2164-12-451

Wei Z, Zhu SX, Van den Berg RG, Bakker FT, Schranz ME (2017) Phylogenetic relationships within *Lactuca* L. (Asteraceae), including African species, based on chloroplast DNA sequence comparisons. Genet Resour Crop Evol 64:55-71. doi:10.1007/s10722-015-0332-5

Witsenboer H, Michelmore RW, Vogel J (1997) Identification, genetic localization, and allelic diversity of selectively amplified microsatellite polymorphic loci in lettuce and wild relatives (*Lactuca spp.*). Genome 40:923-936. doi:10.1139/g97-119

Wright S (1978) Evolution and the genetics of populations: Variability within and among natural populations. University of Chicago Press, Chicago, USA.

Yeh FC, Yang R, Boyle T, Ye Z, Mao JX (1999) POPGENE, version 1.32: Microsoft windo- based free ware for population genetic analysis. Computer program and documentation distributed by University of Alberta and Centre for International Foresty Research, Alberta, Canada

Zhang J, Wu K, Zeng S, da Silva JA, Zhao X, Tian CE, Xia, H, Duan J (2013) Transcriptome analysis of *Cymbidium sinense* and its application to the identification of genes associated with floral development. BMC Genomics 14:279. doi:10.1186/1471-2164-14-279

Chapter II. Genetic diversity of Indian lettuce (*Lactuca indica* Linn.) for morphological traits and agronomic performance in South Korea

Abstract

Identifying genetic basis for the important agronomic traits helps to assist future breeding program for *L. indica* Linn. In the present study morphological characterization, agro-morphological performance and diversity was investigated in 38 accessions. For the morphological study, visual observation was done for qualitative traits of leaf, achene, and inflorescence whereas, nineteen quantitative traits were used for agronomic performance and genetic diversity study. For all characters studied, the genotypic mean square variance was significant, indicating high variability across the accessions. The broad sense heritability (h^2B) ranged from 45.85% (seed length) to 98.59% (total node number) whereas genetic advance as a percentage of the mean (GAM%) ranged from 9.33 (Pappas length) to 191(basal branch). Leaf length, leaf blade width, total branch and basal branch, node number, plant height, basal and median internode distance and seed per capatilium were coupled with high heritability and high genetic advance as percentage mean (GAM) indicating additive gene effect and selection of these

trait based on phenotypic observation is effective for better gain. The duration to bolting, flowering, seed set and seed maturity were linked with high H^2B and moderate GAM% and anticipating improvement in productivity via hybridization and selection. Accession number 55 and 8 have shown superior performance for agricultural and morphological characteristics and could be good material for further research and breeding. In Principal component and cluster analysis, few northern and southern accessions were separated with an admixture of accessions and found weaker than SSR marker based clustering.

Keywords: genetic variability; *Lactuca indica*; morphological variation; morphological traits; population structure; SSR markers.

Introduction

Breeders have been interested in searching the trait of high economic value in wild crops for the improvement of cultivated one (Dempewolf et al. 2017). The crop improvement approach is important because it does not only improve the cultivated crops but also study the possibility of cultivation of wild crops for many aspects, for instance, building biotic and abiotic stress resistant cultivars. For the selection of important trait, a better knowledge of genetic variability and diversity among the available germplasm and genotype is necessary (Bhattarai et al. 2016; Chowdhury et al. 2002; Dempewolf et al. 2017; Ojuederie et al. 2014). Generally, genetic variability is estimated by measuring variation in phenotypic or quantitative and qualitative traits, however, it is limited to characterize the quantitative traits influenced by environmental conditions (Kameswara 2004). Finding important traits using genomic tools is costly for whole accessions. In this regard, the phenotypic study helps to identify the peculiar accessions (van Nocker and Gardiner 2014). Therefore, to devise an appropriate breeding strategy for the genetic improvement, genetic variability study using the various approach including quantitative genetics for phenotypic characters is important (Tuhina-Khatun et al. 2015).

The success of domestication of lettuce depends on non-shattering of the ripe capitulate, non-spineless of the stem, and leaves, formation of head, decrease

in latex content absence of early flowering, shortening of internode and bunching of leaves (Lebeda 2009; Mou 2011; Ryder and Whitaker 1976). *L. indica* is extremely variable in term of phenology and development consenting of annual to perennial, lactiferous herbs or shrubs with distinctly variable leaves, and grow in diverse habitat. However, to the best of our knowledge, very little information was reported for morphological characters and no study have made for genetic variability and agronomic study using morphological traits. Therefore, the objective of present study was to invistigate morphological traits, for analyzing genetic variability and agronomic performance across *L. indica* accessions. In addition, this study also aimed to elucidate genetic relationships among 38 accessions collected across Korea using morphological and simple sequence repeat (SSR) genotyped data (which was invistigated in Chapter-I in this thesis).

Materials and methods

Plant materials

This study uses the information from the same plant material used in the earlier study by Oliya et al. (2018). However, the morphological study requires a number of replications from each accession to achieve quality result. Thus, we used only 41 accessions out of 73 accessions from 41 collection sites where each collection site has equal number of replications (Table II-S1). We excluded the remaining accessions because of the unavailability of replications (because of germination failure). Among the 41 collection site, 30 accessions were collected at a juvenile period in 2015 from their natural habitat; the remaining 11 accessions obtained as seed from the Wild Plant Seed Bank of Korea (http://www.seedbank.re.kr) and Korean National Arboretum (http://www.forest.go.kr) were used. The studied accessions have different areas of origin (Table II-S1). The collected whole plant and seed were planted in Seoul National University experimental farm, Suwon, the Republic of Korea (N 37° 16' 12.094", E 126° 59' 20.756"), for seed harvesting. For morphological characterization, the seed from each accession was sown in the greenhouse on April 29, 2016. The seedling was transplanted 3 weeks later in the field with a row spacing of 60 cm and planting interval of 30 cm in a

randomized complete block design with three replicates. Four competitive plants per block were randomly selected and tagged.

Data Collection

Three accessions, acc. 23, 27, and 28 (Table II-S1) did not enter to the reproductive phase (neither bolt nor flower until the seed harvest and full maturity of other accessions), however, remain in the juvenile period until November 23, 2016. So, these three accessions could not use for further analysis. For remaining 38 accessions, visual observation for leaf shape variation was performed at the flowering time by following the standard evaluation system for wild lettuce (Kristkova et al. 2002). Individuals were categorized and other notable variations such as anthocyanin pigmentation, leaf margin and inflorescence type were noted. For the quantitative traits, total of nineteen characteristics were recorded as follows: at fifty percentage flowering stage three leaves per individuals (at center half of the main stem) were selected and leaf length (LL), leaf tip length (LTL), leaf blade width (LBW), leaf lobe number (LOB); at harvest maturity: basal branches (BB), total branches (TB), plant height (PH), basal stem diameter (BSD), total node number (TNN) basal internode distance (BIND), median internode distance (MIND), based on developmental stage, duration from seeding

to: bolting (BOLT), first flowering (FFD), first seed set (FSD), fifty percent seed maturity (FMD); seed per capitulum/head (SPC), seed length (SL), pappus length (PL), and thousand seed weight (TSW) (Table II-S1).

Data analysis

Analysis of variance

The data collected for nineteen quantitative characters were subjected to analysis of variance (ANOVA) to test the variation among genotypes. ANOVA was performed using R statistical software.

Estimation of variability components

The genotypic variance (GV) is the variance contributed by genetic occurrence of difference among the individuals due to their genetic makeup and the phenotypic variance (PV) is the sum of variance contributed by genetic causes and environmental factors. GV and PV were estimated based on the formula given by (Syukur et al. 2012) as follows,

$$\sigma_g^2 = \frac{[(MSG) - (MSE)]}{r} \quad (1)$$

$$\sigma_p^2 = [\sigma_g^2 + (\frac{\sigma_e^2}{r})] \quad (2)$$

(Where σ_g^2: genotypic variance; σ_p^2: phenotypic variance; σ_e^2: environmental variance (error mean square from the analysis of variance); MSG: mean square of genotypes; MSE: error mean square; r: number of replications)

The genotypic coefficient of variation (GCV) and the phenotypic coefficient of variation (PCV) are the magnitude of genotypic and phenotypic variation existing in a character and were measured by formula suggested by (Burton 1952) as follows,

$$GCV\% = \left[\frac{(\sigma_g)}{X}\right] \times 100 \quad (3)$$

$$PCV\% = \left[\frac{\sigma_p}{X}\right] \times 100 \quad (4)$$

(Where: σ_g : genotypic standard deviation, σ_p: phenotypic standard deviation, X: grand mean of the character)

The heritability estimates the degree of variation in a phenotypic trait in a population that is due to genetic variation between individuals in a population. The Heritability in a broad sense (h^2B) was calculated per formula is given by (Allard 1960) as follows,

$$h^2B = \left[\frac{\sigma_g^2}{\sigma_p^2}\right] \times 100\% \qquad (5)$$

Similarly, Genetic advance (GA) was determined as described by (Johnson et al. 1955) as follows:

$$GA = K \times \sigma_p \times h^2B \qquad (6)$$

(Where K: the selection differential constant (K=2.06 at 5% selection intensity).

The genetic advance as a percentage of the mean (GAM%) also known as the genetic gain was calculated as described by (Johnson et al. 1955) as follow:

$$GAM(\%) = \frac{GA}{X} \times 100 \qquad (7)$$

Quantification of better accessions

Box and whisker plot was performed using SPSS version 23, from which the outlier can be taken as the important accession for a particular trait. And quartile distribution was performed in MS-excel, from which few particular accessions throughout the range of traits can be screened.

Correlation analysis

The simple correlation coefficients between the morphological characters were calculated using the Pearson correlation coefficient using SPSS version 23.

Principal component analysis (PCA) and cluster analysis (CL)

The PCA was performed using a MICROSOFT EXEL add-in statistical package (trial version of XLSTAT-2018 downloaded from https://www.xlstat.com/en/download) and scatter plot of first two PCs were estimated using the same software and phylogenetic tree was computed using Ward (Ward 1963) method based on the distance between the individuals constructed by Euclidean coefficients. Moreover, to make the comparisons of morphological markers with simple sequence repeat (SSR) marker, SSR genotyped data for 38 accessions were pooled from Oliya et al. (2018) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) phylogenetic tree was constructed.

Table II-S1 Accessions used in this study with detailed address origin and collection date

Accession No[x]	Collection site code[y]	Location	Origin[z]	Collection /date
1	Chunam1	317-4, Massanli, Shinpyeong-myeon, Dangjin, Chungnam	Natural field	5/23/2015
3	Chunam2	31-7 Yuguri, Yugu-eup, Gongju-city, Chungnam	Natural field	5/23/2015
4	Chunam3	339-5, Gamam-ri, Chungsan-myeon, Cheongyang-gun, Chungnam	Natural field	5/23/2015
6	Chunam4	1-65 Mount Wakamiri, Seocheon, Seocheon-gun, Chungnam	Natural field	5/23/2015
7	Jebuk1	108 Baeksong-ri, Shinlim-myeon, Gochang-gun, Jeonbuk	Natural field	5/23/2015
8	Jenam1	346-1, Jinyang-ri, Hampyeong-eup, Hampyeong-gun, Jeonnam	Natural field	5/23/2015
11	Jenam2	897, Dongho-ri, Samho-eup, Yeongam-gun, Jeonnam	Natural field	5/23/2015
12	Jenam3	1850-4 Yeongam-gun, Jeonnam	Natural field	5/24/2015
14	Jenam4	661-9, Chungdong-ri, Bupyeong-eup, Boseong-gun, Jeonnam	Natural field	5/24/2015
15	Jenam5	4-1, Doi-dong, Gwangyang, Jeonnam	Natural field	5/24/2015
16	Jenam6	1191-1 Shindori, Yongbang-myeon, Gurye-gun, Jeonnam	Natural field	5/24/2015
19	Jebuk2	243-3, Suejeon-dong, Namwon-city, Jeonbuk	Natural field	5/24/2015
23	Jebuk4	702, Osan-ri, Ohsu-myeon, Imsil-gun, Jeonbuk	Natural field	5/24/2015
25	Chbuk1	974, Gyepcheonri, Jipyeong-eup, Jipyeong-gun, Chungbuk	Natural field	5/24/2015
27	Chbuk2	223 Bok-eup Bok-eun, Bok-eun, Chungbuk	Natural field	5/24/2015
28	Chbuk3	450-1, Noton-ri, Yongsan-myeon, Youngdong-gun, Chungbuk	Natural field	5/24/2015
31	Gynam1	93, Changseon-myeon, Namhae-gun, Gyeongnam	Natural field	5/24/2015
32	Gynam2	713, gyeongnam mil-yangsi san-oemyeon namgili, Gyeongnam	Natural field	5/30/2015
34	Gybuk1	1233-1, Palace Geonjeong-ri, Ulju-gun, Gyeongbuk	Natural field	5/30/2015
36	Gybuk2	467-3, Sanbu-myeon, Gyeongju, Gyeongju, Gyeongbuk	Natural field	5/30/2015
37	Gawon1	75-1, Haoanri Mountain, Hongcheon-eup, Hongcheon-gun, Gangwon	Natural field	6/5/2015
38	Gybuk3	61-2, Samseori, Ganggu-myeon, Youngdeok-gun, Gyeongbuk	Natural field	6/5/2015
39	Gybyk4	223 Sechon-ri, Dankun-myeon, Gyeongbuk	Natural field	6/5/2015
42	Gybuk5	574-9 Maegok-ri, Poongsan-eup, Andong-si, Gyeongbuk	Natural field	6/6/2015
44	Gybuk6	441-11 Daechon-ri, Bonghyun-myeon, Yeongju, Gyeongbuk	Natural field	6/6/2015
45	Gyggi1	0Seongdong-ri, Tonghyeon-myeon, Paju City, Gyeonggi	Natural field	6/6/2015
46	Gyggi2	292 Jeongok-ri, Jeongeup-eup, Yeoncheon-gun, Gyeonggi	Natural field	6/6/2015
51	Gawon2	673-14 Ojiri, Dongsong-eup, Cheolwon-gun, Gyeonggi	Natural field	6/13/2015
54	Chbuk5	Baeun-gun, Chungbuk	Natural field	6/13/2015
52	Gawon4	Hoengseong-gu, Gangwon	Natural field	6/13/2015
55	Gawon3	Yanggu-gun, Gangwon-do	Seed bank2	6/13/2015
58	Gyggi3	Goryeok Arboretum in Soehleok, Pocheon, Gyeonggi	Seed bank 1	9/10/2001
59	Gyggi4	Goryeok Arboretum in Soehleok, Pocheon, Gyeonggi	Seed bank 1	2002

61	Gyggi5	Igeok Elementary School, Icheon-ri, Soheu-eup, Pocheon, Gyeonggi	Seed bank 1	9/12/2005
63	Gynam3	Sangju-myeon, Namhae-gun, Gyeongsangnam-do	Seed bank2	10/19/2006
64	Gynam4	Dae Seong-myeon of Sancheong-gun, Gyeongnam	Seed bank 1	9/24/2004
65	Gybuk7	Jyangyang-myeon, Yeongcheon City, Gyeongbuk	Seed bank2	10/17/2007
68	Jenam7	Dae Mun-Wando, Wando-gun, Jeonnam	Seed bank 1	2012/10/11
69	Chunam5	Boseok-myeon, Seosan-si, Chungnam	Seedbank2	10/22/2009
70	Chunam6	Chungnam	Seed bank 1	10/09/2013
72	Chbuk4	Baeun-gun, Chuncheongbuk-do, Chungbuk	Seed bank 1	10/11/2006

[x]Accession numbers, 23, 27, 28 remain in juvenile stage until 23 November 2016, so these accession were excluded in this research.

[z]for respected GPS coordinate please refer to supplementary table (Table I-S1)

[y]Seed bank1 abbreviated here is Korean National Arboretum (http://www.forest.go.kr) and seed bank 2 is Wild Plant Seed Bank of Korea (http://www.seedbank.re.kr)

Results

Morphological characterization

Morphological studies showed that, there is high polymorphism in leaf shape ranging from the lobed leaf with different degree of the incision to entire where, out of total 38 accessions, 10 accessions (acc.16, 19, 32, 46, 58, 59, 64, 69 and 70) were heterozygous within the accessions and other were homozygous (Table S1, Fig. II-S1A, E, G, H). Total individuals were categorized following the leaf type category proposed by Doležalová et al. (2002) for wild lettuce. Five leaf groups entire, pinnatilobed, pinnatipart, pinnatifid, and pinnatisect existed. In general, lobed leaf (96.93%) was dominating over entire (3.07%) and in lobe leaf, individuals with pinnatifid leaf incision were dominated (73.45%) followed by pinnatisect (17.41%), pinnatilobed (3.07%) and pinnatipart (2.63) (Fig. II-1). Individuals showed entire, undulating and dentate (slight to dense) margin. Among them, the slight dentate type was the most common. Notable variability was recorded in the occurrence of anthocyanin pigmentation in a rosette and flowering leaves and stem, however; this variation was more distinct at rosette stage. Except individuals belonging to three accessions (acc.32, 51, 70) other showed dense anthocyanin distribution in the involucre bract at the full flowering and seed setting stage, where two patterns namely spot and stripes existed. Two

types of inflorescence pyramidal panicles (in the majority of individuals) and compound corymbs panicles were observed only in two individuals belonging to acc. 8. Moreover, in seven different individuals belonging to different accessions (acc. 28, 36, and 68) fused flowering head were observed.

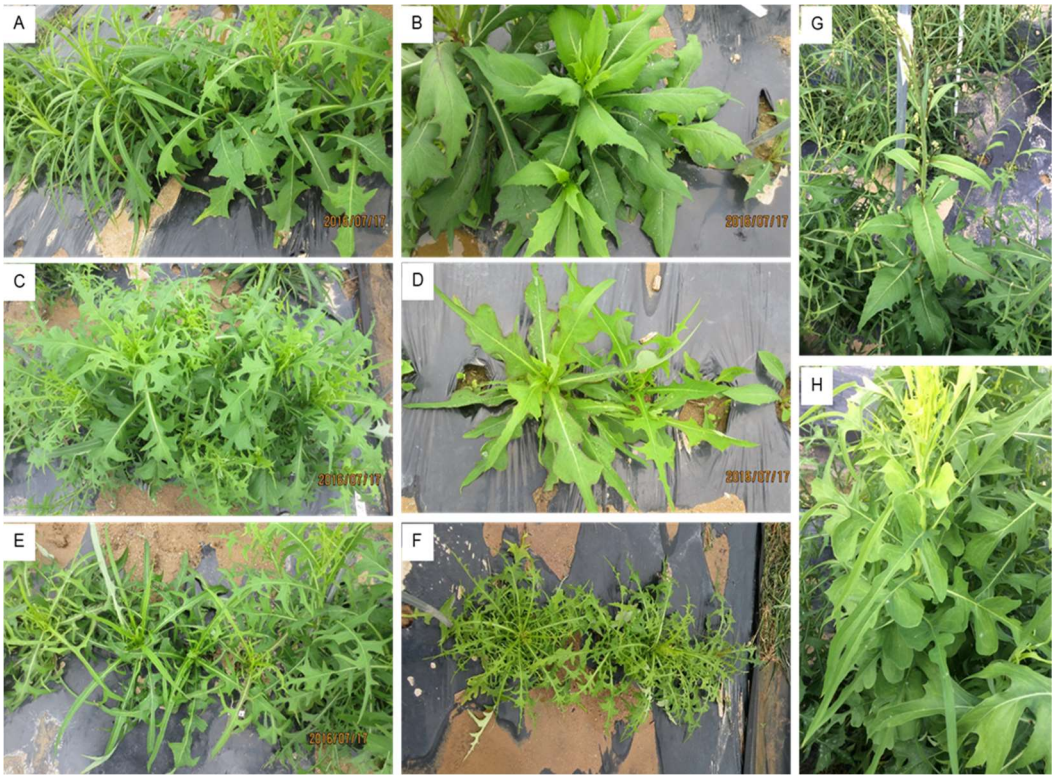

Fig. II-S1 Example of leaf shape variation (intra- and inter- accession) in the studied *L. indica* accessions grown in Suwon. (A-H), some of the representative individuals belonging to different accessions: A, acc. 70; B, G, H, acc.16; C, acc. 6; D, acc.61; E, acc.7; F, acc. 52.

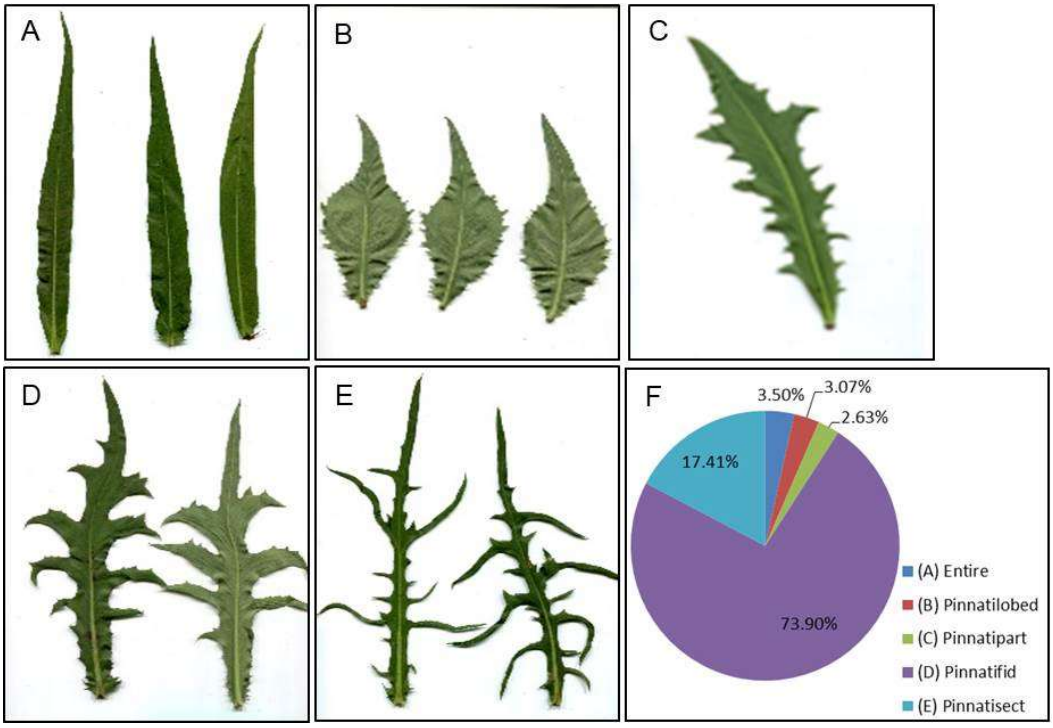

Figure II-1 Variability in leaf character for fully matured cauline leaf from the middle part of stem at a stage of full flowering of *L. indica* accessions collected from South Korea.

Analysis of variance

The extent of variability present in thirty-eight accessions of *L. indica* was measured in term of range, mean plus/minus standard deviation, and the analysis of variance. The individuals showed a considerable range of variation in all studied traits (Table II-1). In ANOVA analysis, the genotypic mean square

variance for all traits was significant. Among the nineteen traits studied, sixteen were significant at P < 0.001, two traits, PL, and TSW were significant at P < 0.01 and only one trait, SL was significant at 0.05 level (Table II-1). This high range of morphological variation with a significant difference between them revealed the presence of ample genetic variability in our accessions.

Table II-1 Different characters used, abbreviations, range, mean, standard deviation and analysis of variance for different quantitative characters in *L. indica*

Traits	Abbreviations	Range	Mean ± SD	MSG[z]	MSE
At flowering					
Leaf length (cm)	LL	9.53-31.00	25.09 ± 4.10	49.97***	3.86
Leaf tip length (cm)	LTL	0.13-21.07	8.06 ± 4.17	51.76***	3.07
Leaf blade width (cm)	LBW	0.52-5.67	1.73 ± 1.05	3.24***	0.17
Leaf lobe number	LOB	0.00-6.33	4.26 ± 1.39	5.7***	1.2
At harvest maturity					
Total branch (number)	TB	16.17-50.33	28.68 ± 6.94	137.75***	47.64
Plant height (cm)	PH	94.33-190.28	143.50 ± 25.11	1870***	144
Basal stem diameter (cm)	BSD	2.00-6.68	4.06 ± 1.04	3.22***	0.95
Total node number	TNN	77.00-287.22	72.62 ± 40.05	4753***	67
Basal branch (number)	BB	0.00-7.00	1.61 ± 1.71	8.67***	1.06
Basal internode distance (cm)	BIND	0.22-3.00	1.53 ± 0.71	1.51***	0.2
Median internode distance (cm)	MIND	0.42-5.13	3.28 ± 1.07	3.38***	0.26
Developmental stage					
Duration to bolting (days)	BOLT	87.00-122.14	108.86 ± 8.06	192.46***	40.44
Duration to first flowering (days)	FFD	105.33-141.42	125.33 ± 9.69	277.9***	40.6
Duration to first seed set (days)	FSD	110.33-149.42	132.31 ± 10.41	322.6***	35.9
Duration to fifty percent seed maturity (days)	FMD	119.94-160.08	142.91 ± 11.36	382.5***	74.8
Seed					
Seed per capitulum (head)	SPC	14.40-28.53	22.1 ± 3.35	33.65***	9.82
Seed length (cm)	SL	0.31-0.46	0.38 ± 0.04	0.01*	0.003
Pappus length (cm)	PL	0.69-0.95	0.85 ± 0.07	0.01**	0.006
Thousand seed weight (gm)	TSW	0.63-2.38	1.12 ± 0.45	0.61**	0.32

*** significant at 0.001, **significant at 0.01, *Significant at 0.05. MSG=Mean square of genotype, MSE= Error mean square

Estimation of variability components

To compare the variation among morphological traits, the estimate of variability component for 19 characters were estimated and presented in Table II-2. The highest and lowest GV and PV was obtained for TNN and TSW, respectively. GCV ranged from 6.11 to 99.12%, and PCV ranged from 7.36 to 105.77%. In general, PCV was found higher than GCV for all traits indicating the role of environment in the expression of character. BB showed the highest value both for GCV and PCV however, PL and BOLT resembled the lowest value. According to Deshmukh et al. (1986), the estimate of GCV and PCV values greater than 20% are regarded as high and values between 10% and 20% to be medium, whereas values less than 10% are considered to be low. In the present study, lower GCV and PCV value were recorded for BOLT, FFD, FSD, FMD, and PL which indicate the influence of environment for trait expression. Other traits were moderate to high for GCV and PCV indicating less influence of environment for expression of these traits. According to Dabholkar (1999), heritability estimates can be placed as low ranging from 5 to 30%; medium ranging from 30 to 60%; and high ranging from 60% to above. From this point of view, the broad sense heritability estimates were medium for SL, PL, TSW and high for other traits, with TNN (98.59%) showing highest and SL (45.85%) showing lowest value. The genetic advance ranged from 0.08 (PL) to 80.84 (TNN). Genetic advance as a

percentage of mean (GAM%) ranged from 9.33% (PL) to 191.35% (BB). The GAM% can be categorized according to Jonhson et al. (1955): low (< 10%), moderate (10–20%) and high (> 20%) in this context, GAM% low for SL and PL, moderate for BOLT, FFD, FSD, FMD, SL and was high for other traits. The top three genetic gains (=GAM%) were obtained for BB (191.35%) followed by LBW (117.04%) and TNN (111.31%), respectively (Table II-2).

Table II-2 Estimate of genetic parameters for evaluated traits in *L. indica*

Characters[x]	GV	PV	GCV %	PCV %	h^2B%	GA	GAM%
LL	15.47	16.66	15.63	16.27	92.28	7.76	30.92
LTL	16.23	17.25	49.96	51.51	94.07	8.05	99.82
LBW	1.02	1.08	58.37	59.96	94.75	2.03	117.04
LOB	1.50	1.90	28.71	32.32	78.92	2.24	52.54
TB	30.04	45.92	19.11	23.63	65.42	9.13	31.84
PH	575.33	623.33	16.72	17.40	92.30	47.47	33.08
BSD	0.76	1.07	21.46	25.53	70.64	1.51	37.15
TNN	1562	1584.33	54.42	54.81	98.59	80.84	111.31
BB	2.54	2.89	99.12	105.77	87.82	3.08	191.35
BIND	0.43	0.50	43.23	46.47	86.55	1.26	82.85
MIND	1.04	1.13	31.08	32.34	92.36	2.02	61.52
BOLT	50.67	64.15	6.54	7.36	78.99	13.03	11.97
FFD	79.10	92.63	7.10	7.68	85.39	16.93	13.51
FSD	95.57	107.53	7.39	7.84	88.87	18.98	14.35
FMD	102.57	127.5	7.09	7.90	80.44	18.71	13.09
SPC	7.94	11.22	12.75	15.15	70.82	4.89	22.11
SL	0.001	0.002	7.34	10.84	45.85	4.00	10.24
PL	0.003	0.005	6.11	8.25	54.85	0.08	9.33
TSW	0.09	0.20	27.54	39.97	47.49	0.44	39.10

[x]LL: leaf length, LTL: leaf tip length, LBW: leaf blade width, LOB: leaf lobe number; BB: basal branch, TB: total branch, PH: plant height, BSD: basal stem diameter, TNN: total node number, BIND: basal internode distance, MIND: median internode distance, BOLT duration to bolting , FFD: duration to first flowering, FSD: duration to first seed set, FMD: duration to fifty percent seed maturity, SPC: seed per capatilium, PL: pappus length, SL: seed length, TWS: thousand seed weight, GV: genotypic variance, PV: phenotypic variance, GCV: genotypic coefficient of variance, PCV, phenotypic coefficient of variance, H^2B: broad sense heritability, GA: genetic advance, GAM: genetic advance as percentage of mean.

Quantification of better accessions

Box and whisker plots were used to visualize distributions of values for each trait across the accessions, where the line within the box represents for the median, the box range includes the second and third quartile, and the whiskers represent the minimum and maximum values. The outliers are represented in the hallowed circle and the star. The Box-whisker plot of agronomic performance (Fig II-2) showed the distribution of accessions within the normal distribution range for the majority of traits however, some accessions distributed in the form of outliers are the potential accessions for particular traits and could be used for future breeding improvement of the particular traits using crossing and hybridization study (Fig.II-2).

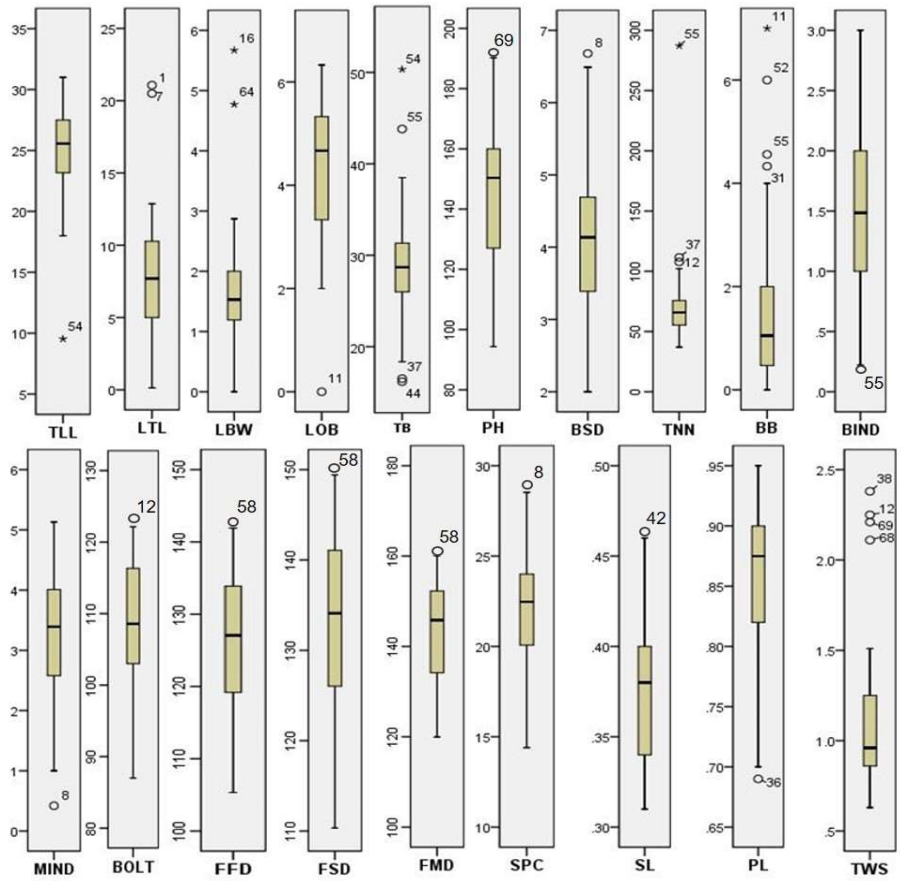

Figure II-2 Box-whisker plot of agronomic performance for recorded traits observed for 38 accessions, the outlier accessions are potential donor accessions for particular trait. The black horizontal line represents the median value, the boxes represents the middle quartiles, the whiskers represents the range of data and the hallo points and stars represents the outliers. Abbreviations, LL: leaf length, LTL: leaf tip length, LBW: leaf blade width, LOB: leaf lobe number; BB: basal branch, TB: total branch, PH: plant height, BSD: basal stem diameter, TNN: total node number, BIND: basal internode distance, MIND: median internode distance BOLT: duration to bolting, FFD: duration to first flowering, FSD: duration to first seed set, FMD: duration to fifty percent seed maturity, SPC: seed per capatilium, PL: pappus length, SL: seed length, TWS: thousand seed weight.

Moreover, to quantify the particular accessions throughout the range of traits we plotted them into the quartile distribution and quartile member sets were identified based on the economic importance of agronomic traits. Higher values were expected for all traits except for LTL, LOB, BIND, and MIND, where a lower value is considered better for greater economic gain. Thus, for high measuring traits fourth quartile and first quartile for low measuring traits were taken. With this assumption, two accessions acc. 8 and 55 are superior whose overall trait value in the fourth quartile are 12 and 11, respectively (Table II-S2)

Table II-S2 Accession membership to quartile in evaluated morphological traits

Acc.	TLL[x]	LTL	LBW	LOB	TB	PH	BSD	TNN	BB	BIND	MIND	BOLT	FF	FSD	FMD	SPC	SL	PL	TSW	Quartile count			
																				Q1	Q2	Q3	Q4
1	Q4	Q4	Q2	Q1	Q4	Q1	Q3	Q1	Q4	Q2	Q2	Q1	Q2	Q2	Q3	Q1	Q2	Q3	Q1	6	6	3	4
3	Q1	Q3	Q3	Q1	Q2	Q2	Q1	Q1	Q4	Q4	Q2	Q1	Q1	Q1	Q1	Q1	Q4	Q2	Q3	9	4	3	3
4	Q2	Q4	Q2	Q1	Q4	Q2	Q2	Q3	Q1	Q4	Q2	Q4	Q3	Q4	Q4	Q1	Q3	Q2	Q3	3	6	4	6
6	Q3	Q3	Q2	Q4	Q4	Q3	Q4	Q2	Q3	Q1	Q2	Q1	Q1	Q1	Q1	Q1	Q4	Q2	Q1	7	4	4	4
7	Q4	Q4	Q3	Q1	Q3	Q2	Q3	Q3	Q3	Q2	Q3	Q3	Q3	Q2	Q2	Q3	Q4	Q4	Q3	1	4	10	4
8	Q3	Q4	Q1	Q2	Q3	Q2	Q4	Q4	Q4	Q4	Q4	Q4	Q4	Q4	Q3	Q4	Q4	Q4	Q3	1	2	4	12
11	Q4	Q1	Q1	Q1	Q2	Q2	Q2	Q4	Q4	Q4	Q3	Q4	Q3	Q3	Q3	Q1	Q1	Q4	Q3	5	3	5	6
12	Q4	Q3	Q3	Q1	Q2	Q4	Q2	Q4	Q2	Q3	Q3	Q4	Q4	Q4	Q4	Q2	Q1	Q4	Q4	2	4	4	9
14	Q2	Q2	Q4	Q3	Q1	Q1	Q1	Q1	Q1	Q1	Q1	Q2	Q3	Q3	Q4	Q1	Q3	Q1	Q2	9	4	4	2
15	Q2	Q3	Q3	Q3	Q2	Q4	Q4	Q2	Q4	Q2	Q2	Q3	Q3	Q3	Q3	Q2	Q1	Q4	Q2	1	7	7	4
16	Q1	Q2	Q4	Q4	Q4	Q4	Q2	Q2	Q2	Q2	Q3	Q3	Q4	Q4	Q4	Q2	Q3	Q2	Q2	1	8	3	7
19	Q1	Q1	Q2	Q2	Q1	Q1	Q1	Q1	Q4	Q1	Q2	Q2	Q2	Q2	Q2	Q4	Q3	Q2	Q2	7	9	1	2
25	Q4	Q3	Q3	Q3	Q2	Q3	Q3	Q4	Q1	Q3	Q4	Q1	Q2	Q3	Q2	Q1	Q2	Q3	Q2	3	5	8	3
31	Q2	Q2	Q4	Q1	Q1	Q1	Q1	Q3	Q4	Q4	Q4	Q4	Q3	Q3	Q3	Q1	Q4	Q4	Q4	5	2	4	8
32	Q1	Q3	Q4	Q2	Q1	Q4	Q2	Q2	Q3	Q4	Q1	Q3	Q3	Q4	Q4	Q4	Q1	Q2	Q2	4	5	4	6
34	Q1	Q1	Q4	Q3	Q2	Q1	Q1	Q1	Q4	Q1	Q4	Q4	Q2	Q3	Q3	Q2	Q2	Q3	Q2	6	5	4	4
36	Q1	Q1	Q4	Q3	Q3	Q3	Q4	Q4	Q1	Q4	Q4	Q3	Q4	Q4	Q3	Q3	Q1	Q1	Q4	5	0	6	8
37	Q2	Q1	Q4	Q4	Q1	Q2	Q1	Q4	Q1	Q3	Q4	Q4	Q4	Q4	Q4	Q4	Q1	Q1	Q1	7	2	1	9
38	Q2	Q3	Q2	Q4	Q1	Q1	Q1	Q1	Q1	Q4	Q1	Q3	Q4	Q3	Q4	Q2	Q4	Q4	Q4	6	3	3	7
39	Q1	Q1	Q1	Q2	Q3	Q3	Q4	Q1	Q1	Q4	Q2	Q3	Q4	Q3	Q3	Q3	Q3	Q3	Q3	5	2	9	3
42	Q1	Q2	Q1	Q1	Q4	Q3	Q2	Q3	Q3	Q2	Q3	Q1	Q2	Q2	Q2	Q3	Q4	Q4	Q2	4	7	5	3
44	Q3	Q4	Q3	Q4	Q1	Q1	Q1	Q2	Q2	Q3	Q3	Q3	Q2	Q2	Q2	Q3	Q2	Q3	Q3	3	6	8	2
45	Q2	Q1	Q1	Q4	Q1	Q1	Q3	Q1	Q3	Q1	Q3	Q1	Q1	Q1	Q1	Q3	Q3	Q3	Q1	11	1	6	1
46	Q3	Q1	Q2	Q3	Q4	Q3	Q4	Q2	Q1	Q1	Q1	Q2	Q1	Q1	Q1	Q2	Q2	Q1	Q4	8	5	3	3
51	Q3	Q2	Q1	Q1	Q4	Q2	Q4	Q3	Q1	Q1	Q1	Q4	Q4	Q4	Q4	Q2	Q4	Q1	Q3	6	3	3	7
52	Q4	Q3	Q1	Q3	Q3	Q3	Q3	Q3	Q4	Q4	Q1	Q1	Q1	Q1	Q2	Q4	Q3	Q3	Q2	5	2	8	4
54	Q1	Q1	Q1	Q3	Q4	Q1	Q3	Q2	Q2	Q1	Q4	Q2	Q1	Q1	Q1	Q1	Q1	Q1	Q2	11	4	2	2
55	Q4	Q4	Q1	Q3	Q4	Q4	Q4	Q4	Q4	Q4	Q4	Q3	Q3	Q3	Q4	Q3	Q3	Q3	Q4	1	0	7	11
58	Q3	Q3	Q2	Q2	Q2	Q3	Q2	Q2	Q1	Q1	Q3	Q4	Q4	Q4	Q4	Q4	Q1	Q1	Q1	5	5	4	5
59	Q1	Q2	Q3	Q2	Q3	Q2	Q1	Q1	Q3	Q3	Q2	Q2	Q2	Q2	Q1	Q4	Q1	Q2	Q1	6	8	4	1
61	Q4	Q4	Q2	Q2	Q3	Q4	Q3	Q2	Q2	Q1	Q2	Q1	Q1	Q1	Q1	Q4	Q1	Q3	Q1	7	5	3	4
63	Q4	Q4	Q3	Q1	Q1	Q3	Q1	Q3	Q3	Q2	Q3	Q1	Q1	Q1	Q1	Q3	Q2	Q3	Q1	8	2	7	2
64	Q3	Q2	Q4	Q1	Q1	Q1	Q1	Q1	Q3	Q2	Q1	Q2	Q3	Q2	Q2	Q1	Q4	Q3	Q4	7	5	4	3
65	Q2	Q1	Q3	Q2	Q4	Q4	Q2	Q3	Q2	Q4	Q1	Q2	Q2	Q2	Q2	Q4	Q3	Q1	Q1	4	8	3	4

68	Q3	Q4	Q2	Q1	Q3	Q4	Q3	Q4	Q2	Q4	Q1	Q1	Q1	Q1	Q1	Q2	Q1	Q2	Q4	7	4	3	5
69	Q3	Q4	Q3	Q2	Q2	Q4	Q4	Q4	Q2	Q2	Q1	Q2	Q2	Q2	Q2	Q4	Q2	Q1	Q4	2	9	2	6
70	Q4	Q2	Q1	Q1	Q2	Q2	Q3	Q4	Q3	Q3	Q4	Q4	Q3	Q4	Q3	Q2	Q2	Q1	Q4	3	5	5	6
72	Q2	Q2	Q2	Q4	Q3	Q3	Q4	Q3	Q2	Q2	Q4	Q2	Q1	Q1	Q1	Q2	Q2	Q2	Q1	4	9	3	3

[x]LL: leaf length, LTL: leaf tip length, LBW: leaf blade width, LOB: leaf lobe number, BB: basal branch , TB: total branch, PH: plant height, BSD: basal stem diameter, TNN: total node number, BIND: basal internode distance, MIND: median internode distance; BOLT: duration to bolting, FFD: duration to first flowering, FSD: duration to first seed set, FMD: duration to fifty percent seed maturity, SPC: seed per capitulum (head), SL: seed length, PL: pappus length, TWS: thousand seed weight

Correlation analysis

Leaf length showed significant positive correlation with leaf tip length (LTL) ($r = 0.462$, $P < 0.01$), and significant negative correlation with LOB ($r = -0.373$, $P < 0.05$). BB showed negative correlation with LOB ($r=-0.325$) and BIND ($r = -354$) however, the correlation of BB was positive with PL ($r = 0.389$). The correlation of TB with BSD and TNN was $r= 0.573$ and 0.36, respectively. PH was positively correlated with BSD ($r= 0.495$, $P< 0.001$), TNN ($r = 0.363$, $P < 0.05$) and SPC ($r = 0.326$, $P < 0.05$). In addition, TNN showed strong negative correlation with BIND ($r = -0.439$) and MIND ($r= -0.453$) but the correlation with TB, PH and BSD was positive ($r = 0.361$, 0.363 and 0.459, respectively). Interestingly, the reproductive traits such as BOLT, FFD, FSD and FMD were strongly positively correlated with each other, however these traits showed no correlation with other traits. Where the correlation of BOLT with FF ($r=0.92$, $P<0.01$), FSD ($r=0.896$, $p<0.01$) and SMD ($r=0.827$, $p<0.01$). Similarly, correlation of FSD with FFD ($r= 0.967$, $P<0.01$) and FMD ($r=0.922$, $P<0.01$) and FMD with FSD was ($r=0.936$, $P<0.01$). PL showed certain positive correlation with TB and SL (correlation of PL with TB and SL= 0.389 and 0.383 at $p = 0.05$) and TSW showed no correlation with any of the traits studied (Table II-3).

Table II-3 Coefficient of correlation among the morphological characters in the studies accessions of *L. indica*

Traits[x]	LL	LTL	LBW	LOB	TB	PH	BSD	TNN	BB	BIND	MIND	BOLT	FFD	FSD	FMD	SPC	SL	PL	TSW
LL	1.00																		
LTL	0.462**	1.00																	
LBW	-0.219	-0.021	1.00																
LOB	-0.373*	-0.162	0.206	1.00															
TB	-0.206	0.162	-0.274	-0.093	1.00														
PH	0.270	0.177	-0.071	-0.077	0.240	1.00													
BSD	0.244	0.231	-0.410*	0.078	0.573**	0.495**	1.00												
TNN	0.187	0.125	-0.099	0.029	0.361*	0.363*	0.459**	1.00											
BB	0.124	-0.134	-0.222	-0.325*	0.097	-0.159	0.000	0.213	1.00										
BIND	-0.248	-0.008	0.062	0.236	0.158	-0.185	-0.084	-0.439**	-0.354*	1.00									
MIND	0.082	0.023	-0.009	-0.129	-0.199	0.179	-0.192	-0.453**	-0.168	0.183	1.00								
BOLT	0.086	-0.176	0.082	-0.059	-0.046	0.047	0.029	0.265	-0.078	-0.212	-0.291	1.00							
FFD	0.042	-0.117	0.215	-0.078	-0.098	0.090	-0.014	0.229	-0.120	-0.258	-0.216	0.927**	1.00						
FSD	0.040	-0.089	0.205	-0.105	-0.109	0.104	-0.001	0.185	-0.141	-0.245	-0.248	0.896**	0.967**	1.00					
FDM	0.033	-0.089	0.206	-0.066	-0.174	-0.009	-0.105	0.181	-0.013	-0.253	-0.151	0.827**	0.922**	0.936**	1.00				
SPC	0.134	0.105	-0.140	0.212	-0.122	0.326*	0.248	0.175	-0.130	-0.189	-0.058	0.143	0.114	0.110	0.064	1.00			
SL	0.052	0.179	-0.015	-0.119	0.031	-0.272	-0.029	-0.060	0.105	-0.044	-0.014	-0.095	0.002	-0.002	0.053	-0.182	1.00		
PL	0.259	0.268	-0.061	-0.225	-0.033	-0.153	0.031	0.013	0.389*	-0.308	-0.110	-0.099	-0.138	-0.124	-0.090	-0.090	0.383*	1.00	
TSW	0.166	0.060	0.029	-0.192	-0.072	0.172	0.009	0.163	-0.171	-0.208	0.226	0.280	0.255	0.183	0.173	-0.029	0.047	0.102	1.00

**Correlation is significant at 0.05 and ** correlation is significant at 0.01 level.*

[x]LL: leaf length, LTL: leaf tip length, LBW: leaf blade width, LOB: leaf lobe number, BB: basal branch, TB: total branch, PH: plant height, BSD: basal stem diameter, TNN: total node number, BIND: basal internode distance, MIND: median internode distance; BOLT: duration to bolting, FFD: duration to first flowering, FSD: duration to first seed set, FMD: duration to fifty percent seed maturity, SPC: seed per capitulum (head), SL: seed length, PL: pappus length, TWS: thousand seed weight.

Principal component analysis (PCA)

In the PCA, 64% of total phenotypic variation was explained by first five components (Table II-4). The first component (PC1) accounted for 22.09% of phenotypic variation where the reproductive traits such as BOLT, FFD, FSD, FMD have high loading. The second component (PC2) explained additional 15.29% of phenotypic variation, which featured by BSD, PH, TB, TNN. Similarly, LOB accounted for the highest score in third component (PC3) which covered additional 11.86% of phenotypic variation. The principal component fourth (PC4) and five (PC5) accounted for additional 9.65% and 7.11% of the phenotypic variation. The highest eigenvalue was covered by PC1 (4.20) in decreasing order followed by PC2, PC3, PC4, and PC5, respectively (Table II-4).

Table II-4 Principal component analysis for 19 quantitative traits across 38 *L. indica* accessions

Traits	PC1	PC 2	PC 3	PC 4	PC 5
Leaf length	0.06	0.31	-0.21	0.36	-0.09
Leaf tip length	-0.04	0.27	-0.08	0.27	0.28
Leaf blade width	0.08	-0.30	-0.02	0.06	0.02
Lobe number	-0.05	-0.18	0.37	-0.15	-0.15
Total branch number	-0.05	0.29	0.23	-0.29	0.47
Plant height	0.09	0.30	0.30	0.31	-0.05
Basal stem diameter	0.03	0.43	0.29	-0.07	0.16
Total node number	0.19	0.36	0.13	-0.21	-0.09
Basal branch number	-0.03	0.19	-0.34	-0.34	-0.26
Basal internode distance	-0.20	-0.24	0.24	0.03	0.42
Midian internode distance	-0.15	-0.11	-0.04	0.51	0.06
Duration to bolting	0.45	-0.05	0.02	-0.04	0.06
Duration to first flowering	0.47	-0.09	0.00	0.00	0.10
Duration to first seed set	0.47	-0.09	0.00	0.00	0.11
Duration to 50% seed maturity	0.44	-0.12	-0.08	-0.02	0.07
Seed per capatilium	0.10	0.15	0.25	0.16	-0.43
Seed length	-0.02	0.04	-0.34	-0.09	0.37
Pappus length	-0.04	0.20	-0.45	-0.07	0.00
thousand seed weight	0.15	0.07	-0.10	0.34	0.15
Eigenvalue	4.20	2.90	2.25	1.83	1.35
Variability (%)	22.09	15.29	11.86	9.65	7.11

In the PCA bi-plot, the majority of accessions from southern province Jeonnam (except one) separated in the right half of the PCA biplot and accessions from the northern province Gyeonggi (except Acc. 55) and all accessions from central Chungnam, grouped in the left half of the PCA. Accessions from Gangwon (except Acc. 55), Chungnam, Gyeonbuk, Gyeongnam, and Jeonbuk distributed in both halves of the PCA (Fig. II-3).

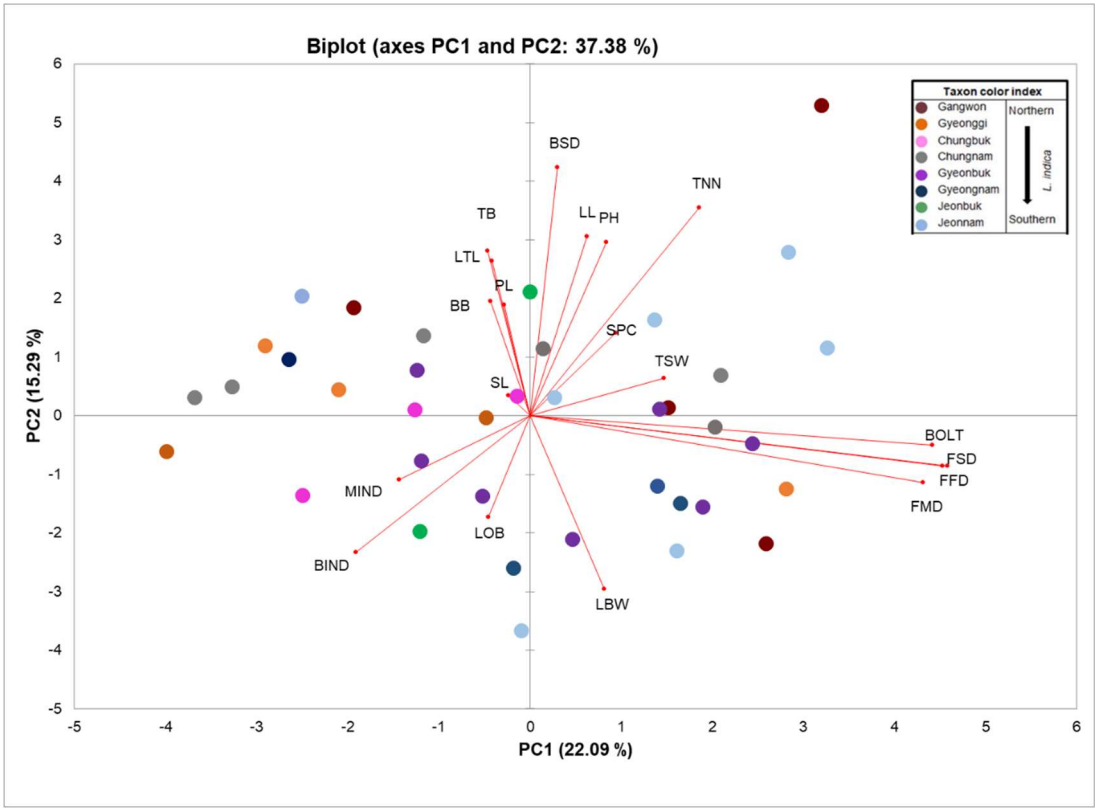

Figure II-3 Principal component bi-plot showing distribution of accessions using nineteen morphological traits.

Cluster analysis (Comparison of morphological traits and SSR marker)

Using morphological traits, accessions were distributed in three different clusters at Euclidean distances of 160. Majority of accessions from southern province Jeonnam (except acc. 14 and 68) grouped in cluster one (CL-I) with an admixture of accessions, however, among two accessions of Jeonbuk, one (acc.7) fell in Cl-I and another grouped in CL-III. Out of five accessions of northern Gyeonggi, three accessions (acc. 46, 59, 61) were grouped in CL-II, one (acc. 58) and another (acc. 45) distributed in CL-I and CL-III, respectively with an admixture of accessions. Similarly, two accessions (acc.37 and 51) and one accession (acc. 52) from Gangwon distributed in CL-I and CL-II, respectively. Interestingly, one accession (acc. 55) of Gangwon isolated in the form of an outlier. In CL-III nine central southern accessions (except Acc. 45 from Gyeonggi) were grouped. In general, accessions did not form any particular clustering according to leaf shape variation, however, one accession (acc. 55) isolated far from other accessions, which showed good performance for 11 quantitative characters (Table II-S2). The most significantly distinct feature for this accession was high node number (Fig. II-2). Another accession acc. 8 which we selected as superior accession from quartile distribution, made closer relation with acc. 36, 37 and 12 in the outer end of CL-I (Fig. II-4A).

In the SSR marker based UPGMA tree, two clusters could be visualized. Where all accessions from central-northern province Gangwon, Gyeonggi and Chungbuk (except acc.25) were grouped in Cl-I and accessions, from southern Jeonnam (except acc.65) and Jeonbuk lies in CL-II. Other accessions from central province Chungnam, Gyeobbuk, and Gyeongnam distributed in both clusters (Fig. II-4B). Hence, SSR marker based phylogenetic tree was more obvious than the cluster analysis of morphological characters to group geographically closer accessions together.

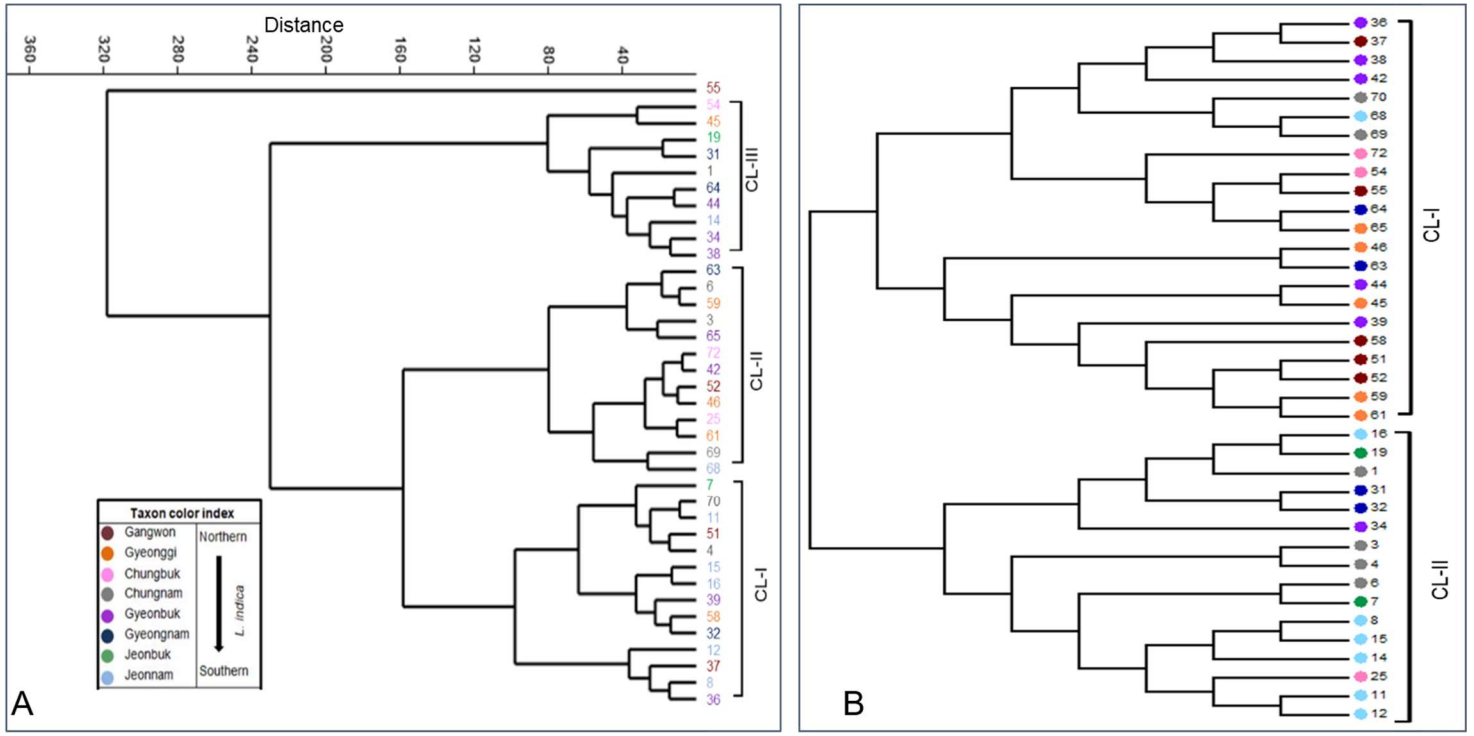

Figure II-4 Phylogenetic relationship between 38 accessions of *L. indica*. A: Wards' phylogenetic tree using Euclidian distance matrix based on 19 morphological traits, B: UPGMA tree drawn using Nei's genetic distance of SSR genotyped data [obtained from Oliya et al. (2018)].

Discussion

In the present study we found presence of ample variability among the studied *L. indica* accessions using morphological (both qualitative and quantitative) characters which showed consistency with the high level of genetic diversity revealed by genic-SSRs in our previous study (Oliya et al. 2018). The genetic variability among *L. indica* individuals is an example of genetic reshuffling, gene flow, natural selection, environmental heterogeneity, and variation in secondary metabolites. As reported in our current research variation in morphology including leaf shape, margin, size distribution of anthocyanin, and duration to bolting and flowering have also reported in other *Lactuca* species including *L. sativa, L. serriola, L. saligna*, *L. Canadensis*, *L. acunetia* (Beharav et al. 2008; Beharav et al. 2010; Doležalová et al. 2002; Lebeda et al. 2010; Lebeda et al. 2009; Ogbodo et al. 2010; Singh 2006). The leaf shape variation is known to play a key role to shape the plants functioning and taxonomical identification (Lindqvist 1958; Niinemets 2015; Wright et al. 2005; Wright et al. 2004). In lettuce breeding, high variation in flowering time has additional economic importance because at flowering time it produces high concentration of sesquiterpene lactone. So early-flowering accessions can be useful for speeding up generation time in genetic, phytomedicinal and growth study (Choi et al. 2016); whereas late-flowering accessions are useful for leaf harvesting for

vegetable purpose. Phenotypically and genetically diverse germplasm is a potentially valuable source for the improvement of a desired growth and reproductive traits including yield, quality and environmental stress tolerance (Zhang et al. 2018). Therefore, screening of accession having better traits are important. In addition, finding important traits using genomic tools is costly for whole accessions. In this regard, the phenotypic study helps to identify the peculiar accessions (van Nocker and Gardiner 2014). In this study, we have screened the potential accessions that results in better fitness for agronomic characters which could allow for replicated and more intensive studies, such as parental selection for breeding, introgression of gene to cultivated lettuce, gene diversity and marker-trait associateion studies (Uwimana et al. 2012). In a similar context, significant marker-trait associations for anthocyanin distribution in leaf and stem, leaf blistering, leaf undulation, leaf color, bolting date and flowering date and the genes responsible for flowering and anthocyanin distribution in lettuce have made available (Kwon et al. 2013; Ryder and Milligan 2005; Zhang et al. 2017).

In a population, observed variation is due to both genetic and environmental factor, however, only the genetic variation is inherited from generation to next generation. Therefore, to devise an appropriate breeding strategy and to predict the maximum genetic improvement for a particular trait that can be achieved through selection, the estimations of genetic parameters like

heritability and genetic advance are needed. The heritability estimates help the breeders in selection based on the basis of phenotypic performance, however, heritability estimate itself cannot give the idea about the expected gain in next generation. Genetic advance is useful in knowing the type of gene action in the expression of different characters, low values of genetic advance showed non-additive gene effects so the selection of characters with low genetic advance might be ineffective. For a best image of the amount of advancement to be expected through phenotypic selection, heritability should be considered in conjugation with genetic advance as a percentage of the mean (Johnson et al. 1995). In the present study, the characters, such as leaf length, leaf blade width, total and basal branch, node number, plant height, basal and median internode distance, seed per capatilium and thousand seed weight were associated with high heritability and high genetic advance as percentage mean indicating additive gene effect and selection of these trait based on phenotypic observation is effective for better gain. Other traits such as the duration for bolting, first flowering, seed set and seed maturity having high heritability were linked with moderate genetic advance as percentage mean and anticipating improvement in productivity via hybridization and selection studies. Moreover, the significant correlation of bolting with flowering, seed set, and seed maturity; node number with plant height, basal stem diameter, seed per capatilium, and internode distance in our

study suggest that the selection practice for one character may simultaneously bring change in the other correlated character.

The PCA and cluster analysis of morphological data also clearly suggest that, acc. 55 is most distinct, however, the method of clustering using morphology is not very stable in compared to the phylogenetic tree of SSR, this could be attributed to the high efficiency of genic-SSR to conserve in the genome than morphological marker which heavily depends on the variable environmental factor. Moreover, the admixture of accessions in both clusters could be attributed to the close proximity of South Korea and the exchange of plant materials across the regions during the history of *L. indica* dispersion. Another reason could be due to the limitation of marker used in the analysis. Therefore, further diversity study using large number of marker set is recommended to investigate better genetic structure. The phenotypic variability is an example of genetic reshuffling, gene flow, natural selection, environmental heterogeneity, and variation in secondary metabolites. In conclusion, the high level of variability and diversity on agro-morphological traits and influence of genetic factor on the majority of trait appeal researcher for the efficient utilization and improvement of *L. indica* in lettuce breeding program.

References

Allard R (1960) Principals of Plant Breeding. John Wiley: New York

Beharav A, Ben-David R, Dolezalova I, Lebeda A (2010) Eco-geographical distribution of *Lactuca aculeata* natural populations in northeastern Israel. Genet Resour Crop Evol 57:679-686 doi:10.1007/s10722-009-9503-6

Beharav A, Ben-DAvid R, Doležalová I, Lebeda A (2008) Eco-geographical distribution of *Lactuca saligna* natural populations in Israel. Isr J Plant Sci 56:195-206

Bhattarai K, Louws FJ, Williamson JD, Panthee DR (2016) Diversity analysis of tomato genotypes based on morphological traits with commercial breeding significance for fresh market production in eastern USA. Aus J Crop Sci 10:1098

Burton GW (1952) Quantitative inheritance in grasses. Proc 6th Int Grassland Cong 1:227-285

Chowdhury M, Vandenberg B, Warkentin T (2002) Cultivar identification and genetic relationship among selected breeding lines and cultivars in chickpea (*Cicer arietinum* L.). Euphytica 127:317-325

Dabholkar A (1999) Elements Of Biometrical genetics (revised And Enlarged Edition). Concept publishing company, New Delhi, India

Dempewolf H, Baute G, Anderson J, Kilian B, Smith C, Guarino L (2017) Past and future use of wild relatives in crop breeding. Crop Sci 57:1070-1082

Deshmukh S, Basu M, Reddy P (1986) Genetic variability, character association and path coefficients of quantitative traits in Virginia bunch varieties of groundnut. Indian J Agric Sci

Doležalová I, Křístková E, Lebeda A, Vinter V (2002) Description of morphological characters of wild *Lactuca* L. spp. genetic resources (English-Czech version). Hort Sci (Prague) 29:56-83

Ha J, Lee T, Kim MY, Oliya BK, Gwag J-G, Lee Y-H, Lee S-h (2017) Comprehensive transcriptome analysis of *Lactuca indica*, a traditional medicinal wild plant. Mol Breed 37:112 doi:10.1007/s11032-017-0711-z

Han Y et al. (2016) MADS-box genes and gibberellins regulate bolting in Lettuce (*Lactuca sativa* L.). Front plant sci 7:1889

Johnson HW, Robinson H, Comstock R (1955) Estimates of genetic and environmental variability in soybeans. Agron J 47:314-318

Kameswara RN (2004). Biotechnology for Plant Resources conservation and use. Principals of seed handling in Genebanks Training course Kampla, Uganda

Kim J-M, Kim J-N, Lee K-S, Shin S-R, Yoon K-Y (2012) Comparison of physicochemical properties of wild and cultivated Lactuca indica. Journal of the Korean Society of Food Science and Nutrition 41:526-532

Kim JM, Yoon KY (2014) Comparison of polyphenol contents, antioxidant, and anti-inflammatory activities of wild and cultivated *Lactuca indica.* Horticulture, Environment, and Biotechnology 55:248-255

Kwon S, Simko I, Hellier B, Mou B, Hu J (2013) Genome-wide association of 10 horticultural traits with expressed sequence tag-derived SNP markers in a collection of lettuce lines. The Crop Journal 1:25-33

Kristkova E, Dolezalova I, Lebeda A, Vinter V, Novotná A (2008) Description of morphological characters of lettuce (*Lactuca sativa* L.) genetic resources. Hortic Sci 35:113-129

Lebeda A, Dolezalová I, Kitner M, Novotná A, Smachova P, Widrlechner M (2010) North American continent-a new source of wild *Lactuca* spp. germplasm variability for future lettuce breeding. In: XXVIII

International Horticultural Congress on Science and Horticulture for People (IHC2010): III International Symposium on 918, 2010. pp 475-482

Lebeda A, Dolezalova I, Kristkova E, Kitner M, Petrzelova I, Mieslerova B, Novotna A (2009) Wild *Lactuca* germplasm for lettuce breeding: current status, gaps and challenges. Euphytica 170:15-34 doi:10.1007/s10681-009-9914-7

Lindqvist K (1958) Inheritance of lobed leaf form in *Lactuca.* Hereditas 44:347-377

McDonald PG, Fonseca CR, Overton J, Westoby M (2003) Leaf-size divergence along rainfall and soil-nutrient gradients: is the method of size reduction common among clades? Funct Ecol 17:50-57

Mizutani T, Liu XJ, Tashiro Y, Miyazaki S, Shimasaki K (1989) Plant regeneration and cell fusion of protoplasts from lettuce cultivars and related wild species in Japan. Bull Fac Agric Saga Univ 67:109–118

Mou B (2011) Mutations in lettuce improvement. Int J Plant Genomics: 1-7

Niinemets Ü (2015) Is there a species spectrum within the world-wide leaf economics spectrum? Major variations in leaf functional traits in the Mediterranean sclerophyll Quercus ilex. New Phytol 205:79-96

Ogbodo E, Okorie P, Utobo E (2010) Growth and yield of lettuce (*Lactuca sativa* L.) at Abakaliki agro-ecological zone of southeastern Nigeria. World J Agri Sci 6:141-148

Ojuederie OB, Balogun MO, Fawole I, Igwe DO, Olowolafe MO (2014) Assessment of the genetic diversity of African yam bean (*Sphenostylis stenocarpa* Hochst ex. A Rich. Harms) accessions using amplified fragment length polymorphism (AFLP) markers. Afr J Biotechnol: 13

Oliya BK, Kim MY, Lee S-H (2018) Development of genic-SSR markers and genetic diversity of Indian lettuce (*Lactuca indica L.*) in South Korea. Genes & Genomics:1-9

Ryder EJ, Whitaker T (1976) Lettuce, *Lactuca sativa* (Compositae). In: NW Simmonds (Ed.). Evolution of crop plants, pp. 39-41. Longman, London

Syukur M, Sujiprihati S, Yunianti R (2012) Teknik pemuliaan tanaman. Penebar Swadaya Jakarta: 348

Thompson RC (1942) An amphidiploid *Lactuca* produced through Non-Reduction in F1 Hybrids. J Hered 33:253-264

Tuhina-Khatun M, Hanafi MM, Rafii Yusop M, Wong M, Salleh FM, Ferdous J (2015) Genetic variation, heritability, and diversity analysis of upland rice

(Oryza sativa L.) genotypes based on quantitative traits. BioMed Res Int 2015

Uwimana B et al. (2012) Hybridization between crops and wild relatives: the contribution of cultivated lettuce to the vigour of crop-wild hybrids under drought, salinity and nutrient deficiency conditions. Theor Appl Genet 125:1097-1111

van Nocker S, Gardiner SE (2014) Breeding better cultivars, faster: applications of new technologies for the rapid deployment of superior horticultural tree crops. Hort Res 1:14022

Ward JH (1963) Hierarchical grouping to optimize an objective function. J Am Stat Assoc 58:236–244

Wei Z, Zhu SX, Van den Berg RG, Bakker FT, Schranz ME (2017) Phylogenetic relationships within *Lactuca* L. (Asteraceae), including African species, based on chloroplast DNA sequence comparisons. Genet Resour Crop Evol 64:55–7

Wright IJ et al. (2005) Modulation of leaf economic traits and trait relationships by climate. Global Ecol Biogeogr 14:411-421

Wright IJ et al. (2004) The worldwide leaf economics spectrum. Nature 428:821

Zhang L et al. (2017) RNA sequencing provides insights into the evolution of lettuce and the regulation of flavonoid biosynthesis Nature communications 8:2264

Zhang Z, Xie W, Zhang J, Zhao X, Zhao Y, Wang Y (2018) Phenotype-and SSR-based estimates of genetic variation between and within two important Elymus species in Western and Northern China. Genes 9:147

Chapter III. *In vitro* propagation, lactucin quantification and antibacterial activity of Indian lettuce (*Lactuca indica* Linn.)

Abstract

Lactuca indica Linn., a member of Asteraceae, is widely used as a traditional medicinal herb. This study develops an efficient protocol for *in vitro* propagation and characterizes the phytomedicinal properties of the plant. The leaf blade and petiole taken from *ex vivo* grown 15 days' plant were cultured on Murashige and Skoog (MS) medium supplemented by the various concentration of 6-Benzylaminopurine (BAP) and Indole-3 acetic acid (IAA). Leaf petiole responded best for direct shoot induction and leaf blade responded best for callus induction. MS medium supplemented by 0.5 mg L^{-1} BAP with 1.2 mg L^{-1} IAA was optimized best for direct shooting and MS medium supplemented by 4 mg L^{-1} BAP and 1.2 mg L^{-1} IAA was best for callus induction. Significantly better callus regeneration (98.67%) was obtained on MS medium supplemented by 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} IAA. MS medium containing 1 mg L^{-1} IAA was the best for *in vitro* rooting. In order to confirm viability of tissue culture in phytomedicinal study, lactucin content and antibacterial activity from in vitro and

naturally grown tissues was investigated. Lactucin was detected in all tissues ranging from 0.2 (tissue culture leaf) to 2.2 µg g^{-}1(juvenileroot). The antibacterial activity of tissue extracts performed against *Pseudomonas fuscovaginae* (rice pathogen) and *Escheriea coli*, resulted in susceptibility where the juvenile root was more effective. In general, root extracts from both tissues by *in vitro* and naturally grown showed better performance for phytomedicinal properties and in antibacterial activity. The established *in vitro* propagation protocol could contribute for large-scale propagation, commercial cultivation, conservation and botanical applications of *L. indica.*

Keywords: agar disc diffusion assay; HPLC analysis; medicinal plant; sesquiterpene lactone; tissue culture.

Introduction

Lactuca indica L. is a lactiferous folklore medicinal herb in the family of Asteraceae, popularly known as Indian lettuce. This plant is widely habitat in Asian countries including South Korea, Indonesia, China, Japan, India, and Malaysia (Jeffrey 1966; Oliya et al. 2018). The leaf of this plant has long been used as a vegetable in the form of salad, soup, fermented food (called "Kimchi" in Korea) and to wrap rice or meat. The leaf of this plant is used as a traditional medicine in the form of tonic to heal fever, cough, diabetics, stomach disorder and for its depurative effect. The plants extract is also used in various medication for its anti-inflammatory, anti-diarrhea, cytotoxicity, hepatoprotective, antibacterial, antidiabetic, antioxidant, α-glucosidase inhibition, immunological parameter enhancer and disease resistance properties (Choi et al. 2016; Harikrishnan et al. 2011; Hou et al. 2003; Kim and Yoon 2014; Kim et al. 2007; Kim et al. 2010; Kim et al. 2008; Lüthje et al. 2011; Park et al. 2014; Wang et al. 2003). Like in other *Lactuca* spp. wounding leaves and stem of this plant release milky latex which becomes sticky when exposed to air; this sticky emulsion helps the plant to defend themselves against herbivorous insects (Agrawal and Konno 2009). The latex is rich in various bioactive compounds such as phenolic and sesquiterpene lactones. Sesquiterpene lactones is the class of terpenoid whose structure is based on guaiane skeleton bearing lactone rings (Graziani et al. 2015).

Various types of sesquiterpene lactones, flavonoids and phenolic compounds have been reported from this plant (Ha et al. 2017; Kim and Yoon 2014; Kim et al. 2008; Michalska et al. 2009; Nishimura et al. 1986). In addition to medicinal and therapeutic properties, it has rich information for breeding as a crop and as a possible candidate to improve traits in cultivated lettuce (Ha et al. 2017; Kim et al. 2012; van Treuren et al. 2013; Oliya et al. 2018).

Increasing demand, anarchic harvesting as well as without proper strategy to replenish the natural resource, medicinal plants like *L. indica* has been diminishing in its natural habitat (Anis and Ahmad 2016; Pant 2013). In nature, *L. indica* regenerates through seed but as in other lettuce, the seed has low viability and dormancy problem when the light requirement for seed germination is offset by low temperature (Eenink 1977; Obembe et al. 2017; Sakpere et al. 2011). In alternative to seed propagation, vegetative propagation is possible, however, the regeneration is limited. In addition, as highlighted by Lebeda et al. (2014), *Lactuca* species are affected by several viral, fungal and bacterial diseases not only in seedling stage but also while transferring in the field. These are the limiting factors to sustainability and large-scale cultivation of this species.

These obstacles could be solved by tissue culture technique, which is widely used for the mass propagation, disease free plant production, genetic improvement, studying and analyzing their secondary metabolites with their pharmaceutical usage (Al Khateeb et al. 2012; Ayan and KevseroĞlu 2007;

Georgiev et al. 2011; Salgotra and Gupta 2016). In addition, this method provides continuous supply of potential resources without any seasonal and environmental limitations, which ensures conservation and their practical application in commercial scale (Kumari et al. 2016). To the best of our knowledge, this is the first report on *in vitro* propagation of *L. indica*. In this study, we develop the protocol for *in vitro* propagation via direct and indirect organogenesis using leaf blade and leaf petiole explant. This study also aimed to quantify the lactucin concentration from *in vitro* developed leaf, callus, root and compare them with the quantities found in different tissues at juvenile and flowering stage of field grown mother plant using high performance liquid chromatography (HPLC). In addition to this, for evaluating the medicinal properties, we also aimed to apply the tissue extracts against two gram negative bacteria, *Pseudomonas fuscovaginae* (a rice pathogen) and *Eschericheia coli*.

Materials and methods

Plant materials culture medium and growth regulators

The plant material used in this study is *L. indica* (acc. 55) used in the previous study by Oliya et al. (2018), which was initially obtained from the National Gene Bank of Korea (http://www.seedbank.re.kr). This accession was one of the potential accessions in the agronomic performance. The mature seed were grown *ex vivo* in soil bed and after 15 days of germination, young areal tissues (leaf blade and petiole) were collected and washed under running tap water for 10 min followed by treatment with liquid detergent (20 ml L^{-1} Tween-20 solution) for 3 min., detergent was washed off and transferred to sterile environment of laminar air flow cabinet. Then rinsed with 70% ethyl alcohol (C_2H_5OH) for 3 min. followed by treatment with 1.5% sodium hypochlorite solution ($NaCl_2$) (v/v) for 10 min. and washed with sterile distilled water for 3 times to remove traces of $NaCl_2$. Then, water was soaked by autoclaved filter paper. Finally, in sterile petri dish, the edge of leaf and mid vein were removed and the remaining part were cut into 0.5 to 0.8 cm in length with the help of sterile blade and forceps.

The basic media composition used for the present study was full strength of Murashige and Skoog (MS) (Murashige and Skoog 1962) medium including vitamins described by Gamborg et al. (1968) and supplemented with 3% (w/v) sucrose. The pH of the medium was adjusted to 5.8 using 1mol L^{-1} sodium hypochlorite (NaOH) and 1mol L^{-1} hydrochloric acid (HCl), before the plant growth regulator were added. Plant growth hormones 6-benzylaminopurine (BAP), indole-3-acetic-acid (IAA) and indole-3-butyric acid (IBA) were used in different concentration and conditions based on the purpose of each study. The medium was gelled with 0.8% (w/v) plant agar. Growth hormones mentioned above were added to media prior to addition of gelling agent. Medium without growth regulator was kept as control. prepared media was autoclaved at 121ºC for 15 min in 15lb pressure. All cultures were kept at (25 ± 2)ºC under a 8: 16 h-dark light per photoperiod using cool white fluorescent light (with 4000 lux) at 23-25˚C during the day and 20˚C at night with 60-70 % relative humidity.

Callus and direct shoot induction

Leaf blade and petiole explants (0.5-0.8) cm^2 in size were inoculated on solidified MS media supplemented with four concentrations (0.5, 1, 2 and 4.0 mg L^{-1}) of BAP with a fixed concentration (1.2 mg L^{-1}) of IAA. MS medium without growth

hormones was used as control. The calli formed from the leaf blade and petiole were periodically sub cultured in every 15 days for multiplication and maintenance. Observation was done for 45 days and callus and shoot bud induction percentage was calculated for each treatment.

Callus multiplication and shoot regeneration

For the purpose of multiple shoot regeneration, healthy calluses (green compact but without shoot bud) were selected, cut into size of 1 cm^2 and transferred to MS media supplemented with four concentrations (0.5, 1, 1.5, and 2.0 mg L^{-1}) of BAP with a fixed concentration (0.5 mg L^{-1}) of IAA. Data recording on percentage of shoot regeneration, number of shoots and shoot length per callus was noted after 45 days of cultured. For measuring shoot length, three longer shoots per calli were selected and mean value were used for analysis.

In vitro rooting, hardening and acclimatization

In vitro regenerated healthy shoots were excised, and transferred to the full strength of MS medium supplemented with 1 and 2 mg L^{-1} of IAA and IBA.

Recording on root number and root length was done for 45 days. For the shoots having more than 3 roots, three longer roots were considered and mean value were used for analysis. From the rooted plants, cap of the culture vessel was opened three days prior to the transplantation. Media was washed with tap water and carefully removed from the root surface and planted in autoclaved greenhouse soil. In addition, they were covered with transparent polythene cap to maintain high humidity around the plants and kept in controlled room. The pots were irrigated with MS half strength salt solution devoid of sucrose for one week. After 7 days, transparent polythene cap was removed 3-4 h daily to expose the plant. After 4 weeks, the plants were transferred under natural conditions and survival rate was accessed.

Extraction of plant tissues

From the *in vitro*, leaf, callus (green color, hard textured), root was collected after 45 days of culture initiation and stored at -20°C. The same accession (acc. no. 55) used for tissue culture, was sown on 14 May 2016 in greenhouse and seedling were transplanted in the field 3 weeks later. At juvenile stage, on 23 July 2016, leaf, root and stem were harvested. At flowering period on 19 September 2016, again root, stem and leaves (at that time plant was in fifty

percent flowering stage) were collected. All collected samples (*in vitro* and naturally grown) were freeze dried for 48 hours after harvesting and stored at 4^{o}C until the further experiment. All stored freeze dried samples were grinded in grinder to fine powder. It is important to notice that, for flowering time root and stem, we separated the bark from the inner hard tissues using mortar and pestle and only the bark was grinded to fine powder.

The extraction was done using the method developed by Willeman et al. (2014) with some modification. Here, 0.1 gram of tissue powder was dissolved in 1.5 ml solvent mixture, water (H_2O)/Chloroform ($CHCl_3$)/ Methanol/CH_3OH (30/30/40, v/v/v), mixed by vortexing. To make homogeneous mixture, 300 µl of additional methanol is added in each tube, vortexed and incubated in water bath at 60°C for one hour and 30 minutes. During the incubation period, the samples were vortexed gently in each 20 minutes' interval. The tubes were agitated for 24h in the dark at room temperature. The tubes were then centrifuged (12,000 r.p.m.at 4°C for 10 min) and supernatant were filter sterilized and stored at -20°C until used for HPLC analysis and bioassays.

Lactucin quantification using high-performance liquid chromatographic (HPLC) analysis

Lactucin was separated using an ultimate 300 HPLC (Thermo Dionex, USA) equipped with a UV-visible Diode Array Detector (190-400nm, DAD scanning and monitored with the Inno-C_{18} column, 4.6mm*250 i.d., 5 µm particles (Youngjinbiochrom, Korea). Buffer A (water 0.1% TFA in distilled water), buffer B (Acetonitrile) mobile phase at a flow rate of 0.8 mL min^{-1} was used. The solution gradient was altered in a linear manner from 95/5, 95/5, 50/50, 10/90, 10/90, 95/5,95/5 (v/v) at 0, 1,30, 31, 36, 37, 40 min., respectively. Dionex™ Chromeleon™ 7.2 (www.thermofisher.com) software was used to calculate the lactucin content. Lactucin (Extrasynthese CS30062-69727 GENAY Cedex, France) was used as standard compound. All the reagent and compound were HPLC grade.

Antibacterial activity assay

L. indica extract was tested for potential antimicrobial activity in agar disc diffusion assay. Bacteria tested were *Pseudomonas fuscovaginae* and *Eschericheia coli*. Both are gram-negative bacteria. *P. fuscovaginae* is soil

bacterium that is pathogenic to rice, causing brown sheath rot. *E. coli* is found in the environment, food, and intestine of animals and humans. Some strain cause diarrhea, urinary tract infection, respiratory illness and pneumonia. These bacteria (concentration of 10^8 CFU$\cdot$ ml^{-1}) were inoculated in the molten Mueller-Hinton agar plate. For agar disc diffusion method, the sterile filter paper disc (diameter 6 mm) was saturated with 40 µl of the tissue extract (10 µl was dispensed at a time and allowed to dry for 10 minutes and repeated for 4 times) then introduced on the upper layer (gently pressed on media) of the bacterial seeded agar plate. For each bacterial strain positive control (ampicillin 10 µl, taken from 1 mg L^{-1}) and negative control, sterile disc concentrated with methanol was used. The plates were incubated overnight at 28 °C for *P. fuscovaginae* and 37 °C for *E. coli*. All plates were examined for the zone of growth inhibition and the diameter of this zone was measured and was compared with standard antibiotic ampicillin and negative control. Three test disc were used in a Petri plate and replicated three times. The result was presented as average.

Statistical analysis

The data were statistically analyzed for significance using one-way analysis of variance (ANOVA) and the differences contrasted using Duncan's

multiple range test (DMRT) at $P \leq 0.05$. All statistical analyses were performed using the SPSS (version 23). The Results were expressed as Mean ± SD of the experiment.

Results

Callus and direct shoot induction

The callus and direct shoots induction using leaf blade and petiole explants are summarized in Table 1. In both cases, the explants became bigger, on the surface of media touched part. In case of the petiole, both callus and direct shooting occurred. Depending on the medium used it took 5-7 days for induction of callus and 4-12 days for adventitious shoot induction (Fig. 1A-D). Among the various concentration of BAP applied on the MS media supplemented by 1.2 mg L^{-1} IAA, 4 mg L^{-1} BAP induced significantly higher (72%) percentage of callus which was followed by 2 mg L^{-1} BAP (55%), 1 mg L^{-1} BAP (25%) and 0.5 mg L^{-1} BAP (10.7%), respectively. Adversely, low BAP concentration (0.5 mg L^{-1}) was significantly good for direct shooting where 87.33% explants induced shoots. Other treatments, 1, 2 and 4 mg L^{-1} BAP induced 69.3%, 12%, and 0.3% shoots, respectively (Table III-1; Fig. III-1A-D).

Table III-1 Effects of plant growth regulators on callus and shoot bud induction from leaf petiole and leaf blade after 4 weeks of *in vitro* culture

Explants	Plant growth regulator (mg L^{-1}) in MS medium		Callus induction % (Mean ± SD)[x]	Days for callus induction (Mean ± SD)	Direct shoot induction % (Mean ± SD)	Days for shoot induction (Mean ± SD)
	BAP	IAA				
Leaf petiole	0.5	1.2	10.7 ± 2.9[d*]	5.8 ± 0.9	87.3 ± 1.5[a]	4.9 ± 0.4[a]
	1.0	1.2	25.0 ± 0.7[c]	7.0 ± 1.0	69.3 ± 1.7[b]	6.8 ± 0.9[b]
	2.0	1.2	55.0 ± 2.9[b]	6.2 ± 0.9	12.0 ± 1.2[c]	12.7 ± 0.6[c]
	4.0	1.2	72.0 ± 1.5[a]	5.5 ± 0.8	5.3 ± 0.9[d]	11.7 ± 0.7[c]
Leaf blade	0.5	1.2	72.67 ± 1.5[c]	11.7 ± 0.6	-	-
	1.0	1.2	74.00 ± 3.8[c]	9.7 ± 1.2	-	-
	2.0	1.2	84.67 ± 0.9[b]	11.0 ± 1.2	-	-
	4.0	1.2	94.67 ± 0.9[a]	11.7 ± 2.7	-	-

[x]data are presented as the means ± standard deviations of replications

[*]values followed by dissimilar letters in the same column are significantly different (P=0.05, Duncan's multiple range test).

In the case of leaf blade, it took 9-12 days, for induction of callus. The calli (greenish compact) covered the leaf blade explant on different media treatment after 3 weeks of culture, however, the calli failed to induce the shoot on these media. Among the medium used, 4 mg L^{-1} BAP induced significantly high callus (94.7%) compared with the other BAP concentrations, 2, 1 and 0.5 mg L^{-1} where, 84.7, 74 and 72.7% callus were induced, respectively (Table III-I).

Callus multiplication and shoot regeneration

In this experiment, the effect of different concentration of BAP (0-2 mg L^{-1}) with 0.5 mg L^{-1} IAA was tested for callus regeneration. The initial visual sign of callus regeneration began 2 weeks after the culture of callus. Shoot were regenerated in all hormonal treatment. There was a significant difference in the percentage of shoot regeneration, number of shoot bud multiplication and shoot elongation for each treatment.

The MS medium supplemented by 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} IAA regenerate maximum callus (98.67%), producing an average of 8.5 microshoots per explant with average shoot length of 4.3 cm (Table III-2; Fig. III-2I, J). MS medium supplemented by 1 and 2 mg L^{-1} BAP and 0.5 mg L^{-1} IAA obtained as the second best media for the similar results. Here, 84% callus was regenerated with an average of 5.6 shoots per callus and the shoot length was 3.8 cm. Only 77% callus regenerate shoots on MS medium treated with 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} IAA. An average of 2.1 shoots was proliferated per callus. The length of the longest shoot in this media was 2.1 cm (Table III-2).

Table III-2 Effects of different concentrations of BAP with 0.5 mg L^{-1} of IAA on shoot induction and elongation from callus cultures, number and length of shoots taken after 4 weeks of culture

Plant growth regulator (mg L^{-1}) in MS medium		Shoot regeneration percentage[x] (Mean ± SD)	Number of shoots per callus (Mean ± SD)	Shoot length (cm) (Mean ± SD)
BAP	IAA			
0.5	0.5	77.0 ± 0.6[c*]	2.1 ± 0.2[c]	2.1 ± 0.2[d]
1.0	0.5	84.3 ± 0.4[b]	4.6 ± 0.3[b]	3.8 ± 0.2[b]
1.5	0.5	98.7± 0.7[a]	8.5 ± 0.4[a]	4.3 ± 0.2[a]
2.0	0.5	86.7 ±1.9[b]	3.8 ± 0.2[b]	3.2 ± 0.1[c]

[x]data are presented as the means ± standard deviations of replications

[*]values followed by dissimilar letters in the same column are significantly different (P=0.05, Duncan's multiple range test).

In vitro rooting, hardening and acclimatization

The emergence of roots on micro shoots was detected after15 days of inoculation to rooting media. The effect of hormones on the rooting of shoots was noted by recording the percentage of shoots that produced roots, a number of root per shoot and an average root length. Among the auxin used, all (100%) micro shoots induced root in the media treated with 1 mg L^{-1} of IAA which was followed by 1 mg L^{-1} of IBA (99.5%), 0.5 mg L^{-1} of IAA (96.4%) and 0.5 mg L^{-1} of IBA (92.5%). There was no significant difference between 1 mg L^{-1} of IAA and IBA for percentage root induction, however, 1 mg L^{-1} IAA found

significantly good for other performance where, an average of 7.5 roots with longer root length (3.9 cm) was recorded (Table III-3, Fig. II-1K). Similarly, 0.5 mg L^{-1} IAA and IBA ranked second and third for percentage of root induction and root length measurement but there was not a significant difference between these two media for induction of root number per shoot (Table III-3). The *in vitro* rooted plants showed good growth response in the greenhouse (Fig. III-1L) and successfully acclimatized in the field. All plants were healthy with a morphology similar to that of source plants.

Table III-3 Effects of different concentration of auxins for root induction and elongation

Auxin used	Concentrations ($mg \cdot L^{-1}$) in MS medium	Percentage of root induction[s] (Mean ± SD)	Number of root/shoot (Mean ± SD)	Root length (cm) (Mean ± SD)
IAA	1.0	100.0 ± 0.0[a*]	7.5 ± 0.4[a]	3.9 ± 0.1[a]
	0.5	96.4 ± 1.0[b]	4.3 ± 0.3[c]	2.7 ± 0.1[c]
IBA	1.0	99.5 ± 0.3[a]	6.3 ± 0.4[b]	3.5 ± 0.1[b]
	0.5	92.5 ± 1.5[c]	3.9 ± 0.24c	2.3 ± 0.1[d]

[x]data are presented as the means ± standard deviations of replications

[y]values followed by dissimilar letters in the same column are significantly different (P=0.05, Duncan's multiple range test).

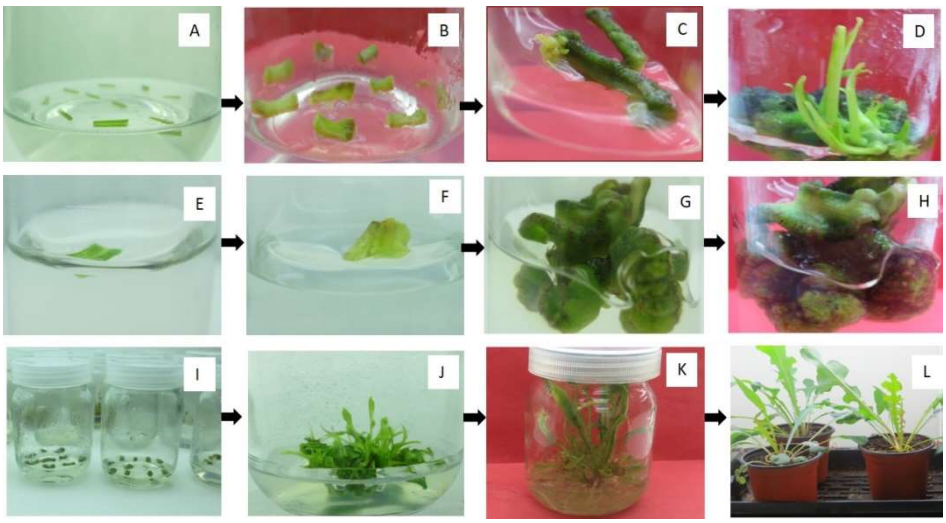

Figure III-1 Regeneration of *Lactuca indica* L. [A-D] petiole explant responded on MS + 0.5 mg L^{-1} BAP + 1.2 mg L^{-1} IAA in different stages of development: (A) petiole explant at the time of inoculation, (B) petiole enlargement after 5 days, (C) induction of shoot buds at 16 days, (D), elongation of shoots at 22 days; [E-F] leaf explant responded on MS + 4 mg L^{-1} BAP + 1.2 mg L^{-1}IAA: (E) leaf explant at the time of inoculation, (F) enlargement at 5 days (G), callus induction at 12 days (H), callus hardening at 22 days; (I-J) callus culture on MS + 1.5 mg L^{-1} BAP + 0.5 mg L^{-1} IAA: (I) sized callus inoculated in the media, (J) shoot regeneration at 30 days; (K), *in vitro* rooted plant on MS +1 mg L^{-1} IAA after 30 days of shoot inoculation to media, (L) well adopted plant in greenhouse after 4 weeks of acclimatization.

Lactucin quantification using HPLC analysis

In HPLC analysis, lactucin (standard 1-Lactucin-17.087) was detected at the retention time of 17 minutes in all tissue extracts and its concentration ranged from 0.2 to 2.2 µg g^{-1} with an average of 0.9 µg g^{-1}. Among the *in vitro* samples, the higher amount of lactucin was obtained from the root (0.9 µg g^{-1}) followed by callus (0.7 µg g^{-1}) and leaf (0.2 µg g^{-1}). From the naturally grown plant, higher concentration of lactucin were detected on root at juvenile stage (2.2 µg g^{-1}) followed by root at flowering stage (1.3 µg g^{-1}), stem at flowering stage (0.8 µg g^{-1}) stem at juvenile stage (0.7 µg g^{-1}), leaf at flowering stage (0.3 µg g^{-1}) and leaf at juvenile stage (0.2 µg g^{-1}), respectively (Fig. III-2A, B). In LSD test, lactucin content in juvenile root was significantly higher and was followed by flowering root. There was not a significant difference between the lactucin amount obtained between tissue cultured root, callus, juvenile stem and flowering stem. Similarly, the lactucin content of tissue cultured leaf, juvenile leaf, and flowering root was significantly lower than other tissue used (Fig. III-2A).

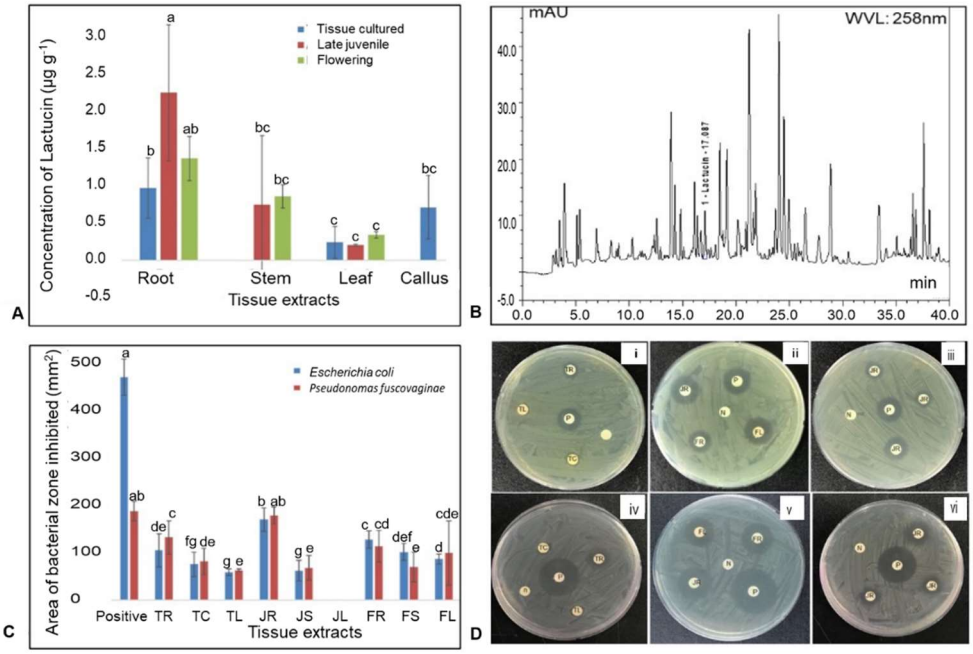

Figure III-2 Characterization of lactucin and its application for antibacterial testing. (A) lactucin concentration in different tissue extracts; (B) chromatogram detecting lactucin in HPLC analysis; (C, D) area of bacterial zone of inhibition (mm^2) shown by different tissue extract against *P. fuscovaginae* (i-iii) and *E. coli* (iv-vi). Abbreviations in bar graph and on the filter paper disc represents, TR: tissue cultured root, TC: tissue cultured callus, TL: tissue cultured leaf, JR: juvenile root, JL: juvenile leaf, JS: juvenile stem, FR: flowering root, FS: flowering stem, FL: flowering leaf, N: negative control solvent mixture (water/chloroform/methanol), P: positive control (ampicillin). The data in the bar graph are presented as the means ± standard deviations of replications, values with dissimilar letters above the error line in the bar graph are significantly different by DMRT at $P \leq 0.05$.

Antibacterial activity

All tissue extracts except a juvenile leaf from the naturally grown parental plant were effective against both bacteria tested (Fig. III-2C, D). Solvent mixture used in negative control did not produce any area of the zone of inhibition. Ampicillin used as positive control showed larger bacterial inhibition area than test samples (465.6 mm^2) for *E. coli.* However, the area of inhibition of positive control (185.7 mm^2) against *P. fuscovaginae* was not significantly different than juvenile root extract (176.5 mm^2).

Among the tissue cultured samples, the root was significantly strong to kill both bacteria. The area of bacterial inhibition of root against *E. coli* and *P. fuscovaginae* were 103.8 mm^2 and 131.0 mm^2, respectively. Using callus extract, bacterial inhibition area of 74.5 mm^2 and 80.7 mm^2 were obtained against *E. coli* and *P. fuscovaginae,* respectively. Leaf extract was less effective to kill both bacteria. (Fig. III-2 C, D). Among the naturally grown samples, except juvenile leaf all showed significant antibacterial effect against both bacteria tested. Juvenile root was significantly strong to kill both bacteria. The effect of juvenile root, flowering root, flowering stem, flowering leaf and juvenile stem against *E. coli* was 168.0, 125.9, 100.3, 85.8 and 61.0 mm^2 in decreasing order, respectively. These value were significantly different by DMRT. For *P. fuscovaginae,* the

inhibition area was obtained as 176.7, 112.1, 69.0, and 67.4 mm^2 in decreasing order for juvenile root, flowering root, flowering stem and flowering leaf, respectively. We could not get any significant difference in the antibacterial activity of juvenile and flowering stem extract for *P. fuscovaginae,* however, there was a significant difference in the antibacterial activity between juvenile and flowering root extract (Fig. III-2C, D).

When compared the effect of tissue cultured samples and naturally grown samples to kill *E. coli*, tissue cultured root was less effective than juvenile and flowering root but more effective than the juvenile and flowering stem. The antibacterial activity of callus was higher than juvenile stem and lower than other extracts. Tissue cultured leaf was not significantly different than juvenile stem but significantly less effective than other extracts. Similarly, for *P. fuscovaginae*, the antibacterial activity of tissue cultured root was significantly less than juvenile root but significantly higher than other extracts. Callus showed significantly lower antibacterial activity than juvenile and flowering root but it showed significantly higher activity than other extracts. The antibacterial activity of tissue cultured leaf was less than root and leaf at juvenile and flowering but not significantly different with flowering and juvenile stem (Fig III-2C).

Discussion

L. indica is being one of the traditional medicinal and edible crop plant, the knowledge of *in vitro* propagation is essential. The development of efficient *in vitro* regeneration system helps for various biotechnological breakthrough including mass propagation, plant genetic transformation metabolites biosynthesis, and somatic hybridization (Chavan et al. 2018). Previously, Mizutani et al. (1989) produced viable callus in somatic hybridization between *L. indica* and *L. sativa*, however, plant regeneration was failed. In the current research, using various concentration of BAP with a fixed concentration (1.2 mgL^{-1}) of IAA, leaf petiole performed best for shoot multiplication both by direct and indirect organogenesis than leaf blade but reverse in callus induction. In addition, a lower concentration of BAP (0.5 mg L^{-1}) significantly increased the shoot induction and multiplication rate and a higher concentration of BAP (4 mg L^{-1}) significantly increased the callus induction rate. Similar results were reported in the tissue culture of cultivated lettuce and Chicory (Armas et al. 2017; Mohebodini et al. 2011; Park and Lim 1997). In addition, they found that the *in vitro* regeneration is genotype dependent. Earlier report on shoot induction of *Launaea taraxacifolia*, an African wild lettuce, showed that BAP was best cytokinin used for shoot induction in combination of NAA (Obembe et al. 2017). In addition, they found that higher concentration of BAP (2.5 mg L^{-1})

significantly increased the shoot induction rate. In our current research, MS medium supplemented by 1.5 mg L^{-1} BAP with 0.5 mg L^{-1} IAA performed best for callus regeneration showed consistency with earlier report on callus regeneration in *Cichorium pumilum* (Wesam et al. 2012) and IAA performed best for *in vitro* rooting than IBA, showed consistency with lettuce and chicory (Dolinski and Olek 2013; Pink and Carter 1987; Yucesan et al. 2007).

A comparative analysis of natural product and *in vitro* bioassay between tissue cultured and naturally grown plant is important to support the utilization of *in vitro* material for pharmacological research and to control dependency in natural population for drug development. Our present result suggests that tissue cultured materials are biochemically potent as the naturally grown plants. In addition, we found high concentration of lactucin in root collected by both *in vitro* and naturally grown plant, however, the entire plant can be used for extraction. The variation in the concentration of lactucin among the tissues may be affected by the surrounding environmental changes during the process of plant development or due to mutations (Badri et al. 2010; Hubbard et al. 2017; Sampaio et al. 2016; Solaiman and Anawar 2015). Previously, Michalska et al. (2009) using ultraviolet (UV)–HPLC and thin layer chromatography (TLC), six different types of sesquiterpene lactones, 8-deoxylactucin, jacquinelin, crepidiaside B, lactucopicrin, glucozaluzanin C and lactuside-A were identified in the root and leaf of *L. indica,* however, they had not detected lactucin both in leaf

and root of this plant. In the recent study, using HPLC method of detection, little amount of lactucin, 8-desoyllactucin, and lactucopricin in leaf at bolting stage and lactucin only at the flowering stage were identified (Ha et al. 2017). Moreover, in the same study lactucin ranging from 1.9 μg g^{-1} to 98.7 μg g^{-1} were characterized in flowering leaf from 61 *L. indica* accessions collected across South Korea. Comparing the work of Ha et al. (2017) with present study we conclude that lactucin synthesis, as well as its concentration in *L. indica* depends on plant organs, phenological development, and the environment.

Regarding the antibacterial activity of different tissue extracts, *P. fuscovaginae* and *E. coli* showed a relatively similar reaction. More interestingly, the juvenile root extract (where significantly higher lactucin concentration was obtained) showed significantly stronger antibacterial activity and the juvenile leaf extract (where low lactucin concentration was obtained) showed no antibacterial activity for both bacteria. Thus, antibacterial activity of tissue extract in our study could be attributed to the presence of lactucin, however, we have not tested the presence of other phytochemicals such as other sesquiterpenoids and phenolic which could present in crude extract as reported by previous studies (Ha et al. 2017; Kim and Yoon 2014; Kim et al. 2008; Michalska et al. 2009; Nishimura et al. 1986). In previous report, Lactucin identified in Chicory showed antibacterial activity against various bacteria (Liu et al. 2013; Nandagopal and Kumari 2007; Petrovic et al. 2004). Similarly, *L. indica* extract showed various therapeutic

activities including antibacterial activity against uropathogenic *E. coli* (Hau et al 2003; Kim et al. 2007; Kim et al. 2010; Lüthje et al. 2011; Park et al. 2014; Wang et al. 2003 (Lüthje et al. 2011). Though therapeutic activities of plant extracts were reported, present study is the first to identify the antibacterial effect of *L.indica* against plant pathogen, *P. fuscovaginae,* which causes brown sheath rot to rice. This result widen the therapuatic horien of this plant. Moreover, no information exist in the literature concerning particular stage at which the tissues should be collected for medicinal purposes. From our result we would strongly recommend to use root by both *in vitro* and naturally grown to use as antibicrobial agent. In addition, while using particular tissue, we recommend to use root collected at juvenile stage; leaves and stem collected at flowering stage.

In conclusion, this study establishes an efficient *in vitro* propagation protocol, quantified lactucin and detect antibacterial activities of tissue extracts both by *in vitro* and naturally grown plant. The result also suggests that plant extracts with antibacterial properties can be used as antimicrobial agents. Our result could contribute for conservation and botanical application of not only *L. indica* but also could be a guide for similar study of other lettuce and medicinal plants.

References

Agrawal AA, Konno K (2009) Latex: a model for understanding mechanisms, ecology, and evolution of plant defense against herbivory. Annu Rev Ecol Evol Syst 40:311-331

Al Khateeb W, Hussein E, Qouta L, Alu'datt M, Al-Shara B, Abu-Zaiton A (2012) In vitro propagation and characterization of phenolic content along with antioxidant and antimicrobial activities of Cichorium pumilum Jacq Plant Cell, Tiss Organ Cult (PCTOC) 110:103-110

Anis M, Ahmad N (2016) Plant tissue culture: propagation, conservation and crop improvement. Springer, Nature, Singapore

Armas I, Pogrebnyak N, Raskin I (2017) A rapid and efficient *in vitro* regeneration system for lettuce (*Lactuca sativa* L.). Plant methods 13:58

Ayan AK, KevseroĞlu K (2007) Direct and indirect regeneration of plants from internodal and leaf expiants of *Hypencum bupleuroides* gris J plant Biol 50:24

Badri DV, Loyola-Vargas VM, Broeckling CD, Vivanco JM (2010) Root secretion of phytochemicals in Arabidopsis is predominantly not influenced by diurnal rhythms. Molecular plant 3:491-498

Chavan J, Gaikwad N, Dixit G, Yadav S, Bapat V (2018) Biotechnological interventions for propagation, conservation and improvement of 'Lantern Flowers'(*Ceropegia spp*.). S Afr J Bot 114:192-216

Choi C-I, Eom HJ, Kim KH (2016) Antioxidant and α-glucosidase inhibitory phenolic constituents of *Lactuca indica* L. Rus J Organ Chem 42:310-315

Dolinski R, Olek A (2013) Micropropagation of wild chicory (*Cichorium intybus* L. var. silvestre Bisch.) from leaf explants. Acta Sci Pol Hort Cultus 12:33-44

Eenink A (1977) Influence of temperature on seed dormancy in lettuce Scientia Horticulturae 6:1-13

Gamborg OLc, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp cell Res 50:151-158

Georgiev V, Ivanov I, Berkov S, Pavlov A (2011) Alkaloids biosynthesis by *Pancratium maritimum* L. shoots in liquid culture Acta physiologiae plantarum 33:927-933

Graziani G, Ferracane R, Sambo P et al (2015) Profiling chicory sesquiterpene lactones by high resolution mass spectrometry. Food Res Int 67:193–198. doi:10.1016/j.foodres.2014.11.021

Ha J, Lee T, Kim MY, Oliya BK, Gwag J-G, Lee Y-H, Lee S-h (2017) Comprehensive transcriptome analysis of *Lactuca indica*, a traditional medicinal wild plant Mol Breed 37:112. doi:10.1007/s11032-017-0711-z

Harikrishnan R, Kim J-S, Kim M-C, Balasundaram C, Heo M-S (2011) Lactuca indica extract as feed additive enhances immunological parameters and disease resistance in Epinephelus bruneus to *Streptococcus iniae* Aquaculture 318:43-47

Hou C-C, Lin S-J, Cheng J-T, Hsu F-L (2003) Antidiabetic Dimeric Guianolides and a Lignan Glycoside from *Lactuca indica*. J Nat Prod 66:625-629

Hubbard CJ, Brock MT, van Diepen LT, Maignien L, Ewers BE, Weinig C (2017) The plant circadian clock influences rhizosphere community structure and function. The ISME J 12:1-11.doi: 10.1104/pp.17.00057

Jeffrey C (1966). Notes on the Compositae: I. The Cichorieae in East Tropical Africa Kew Bull 18:427–486

Kim J-M, Kim J-N, Lee K-S, Shin S-R, Yoon K-Y (2012) Comparison of physicochemical properties of wild and cultivated *Lactuca indica.* J Korean Soc Food Sci Nutr 41:526-532

Kim JM, Yoon KY (2014) Comparison of polyphenol contents, antioxidant, and anti-inflammatory activities of wild and cultivated *Lactuca indica*. Hort Env Biotechnol 55:248-255

Kim KH, Kim YH, Lee KR (2007) Isolation of quinic acid derivatives and flavonoids from the aerial parts of *Lactuca indica* L. and their hepatoprotective activity *in vitro.* Bioorganic Med. Chem. Lett 17:6739-6743

Kim KH, Kim YH, Lee KR (2010) Isolation of hepatoprotective phenylpropanoid from *Lactuca indica*. Nat Prod Sci 16:6-9

Kim KH, Lee KH, Choi SU, Kim YH, Lee KR (2008) Terpene and phenolic constituents of *Lactuca indica* L Archives of pharmacal research 31:983-988

Kumari A, Baskaran P, Van Staden J (2016) *In vitro* propagation and antibacterial activity in Cotyledon orbiculata: a valuable medicinal plant. Plant Cell Tiss Org Cult 124:97-104

Lebeda A, Křístková E, Kitner M, Mieslerová B, Jemelková M, Pink DA (2014) Wild Lactuca species, their genetic diversity, resistance to diseases and pests, and exploitation in lettuce breeding. Eur J Plant Pathol 138:597-640

Liu H, Wang Q, Liu Y, Chen G, Cui J (2013) Antimicrobial and antioxidant activities of *Cichorium intybus* root extract using orthogonal matrix design. J food sci 78: 258-263

Lüthje P, Dzung DN, Brauner A (2011) Lactuca indica extract interferes with uroepithelial infection by *Escherichia coli*. J ethnopharmacol 135:672-677

Michalska K, Stojakowska A, Malarz J, Doležalová I, Lebeda A, Kisiel W (2009) Systematic implications of sesquiterpene lactones in *Lactuca*

Mohebodini M, Mokhtar JJ, Mahboudi F, Alizadeh H (2011) Effects of genotype, explant age and growth regulators on callus induction and direct shoot regeneration of Lettuce (*Lactuca sativa* L.) Aust J Crop Sci 5:92

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473-497

Nandagopal S, Kumari BR (2007) Phytochemical and antibacterial studies of Chicory (*Cichorium intybus* L.)-A multipurpose medicinal plant. Adv Biol Res 1:17-21

Nishimura K, Miyase T, Ueno A, Noro T, Kuroyanagi M, Fukushima S (1986) Sesquiterpene lactones from *Lactuca laciniata*. Phytochemistry 25:2375-2379

Obembe OO, Bello OA, Aworunse OS, Popoola JO, Akposibruke O, Olukanmi BI, Olayode MN (2017) *In vitro* Multiple Shoots Formation in Wild Lettuce (*Launaea taraxacifolia*)(Willd.) Amin ex C. Jeffrey. Annual Research & Review in Biology 19:1-8

Oliya BK, Kim MY, Lee S-H (2018) Development of genic-SSR markers and genetic diversity of Indian lettuce (*Lactuca indica* L.) in South Korea. Genes & Genomics:1-9. doi: 10.1007/s13258-018-0660-x

Pant B (2013). Medicinal orchids and their uses: Tissue culture a potential alternative for conservation. Afr. J. plant sci. 7:448-467

Park E, Lim H (1997) Establishment of an efficient *in vitro* plant regeneration system in chicory (*Cichorium intybus* L. *var. sativus*). In: International Symposium on Vegetable Quality of Fresh and Fermented Vegetables 483, pp 367-370

Park J-H, Shin J-H, Roy SK, Park H-Y (2014) Evaluation of Cytotoxicity, Total Phenolic Content and Antioxidant Innate Reveal Efficient Medications in Native *Lactuca indica*. J Agr Sci 6:135

Petrovic J, Stanojkovic A, Comic L, Curcic S (2004) Antibacterial activity of *Cichorium intybus*. Fitoterapia 75:737-739

Pink D, Carter PJ (1987) Propagation of lettuce (*Lactuca sativa*) breeding material by tissue culture Ann Appl Biol 110:611-616

Sakpere AM, Ayisire ER, Abioye OI (2011) Potential of *Launea taraxacifolia* (Willd) Amin Ex. C. Jeffrey for *in vitro* Regeneration. Notulae Scientia Biologicae 3:93

Salgotra RK, Gupta BB (2015) Plant genetic resources and traditional knowledge for food security. Springer, Berlin. doi:10.1007/978-981-10-0060-7

Sampaio BL, Edrada-Ebel R, Da Costa FB (2016) Effect of the environment on the secondary metabolic profile of Tithonia diversifolia: a model for environmental metabolomics of plants. Sci Rep 6:29265

Solaiman ZM, Anawar HM (2015) Rhizosphere microbes interactions in medicinal plants. In: Plant-Growth-Promoting Rhizobacteria (PGPR) and Medicinal Plants. Springer, pp 19-41

Van Treuren R, Van der Arend A, Schut J (2013) Distribution of downy mildew (*Bremia lactucae* Regel) resistances in a genebank collection of lettuce and its wild relatives. Plant Genet. Resour. 11:15-25

Wang S-Y, Chang H-N, Lin K-T, Lo C-P, Yang N-S, Shyur L-F (2003) Antioxidant properties and phytochemical characteristics of extracts from *Lactuca indica*. J Agr Food Chem 51:1506-1512.

Willeman H, Hance P, Fertin A, Voedts N, Duhal N, Goossens J-F, Hilbert J-L (2014) A method for the simultaneous determination of chlorogenic acid and sesquiterpene lactone content in industrial chicory root foodstuffs. Sci World J 1-11 doi:10.1155/2014/583180

Yucesan B, Turker AU, Gurel E (2007) TDZ-induced high frequency plant regeneration through multiple shoot formation in witloof chicory (*Cichorium intybus* L.). Plant Cell Tiss Org Cult 91:243-250

국문초록

국화과에 속하는 왕고들빼기(*Lactuca indica* L.)은 주요 약용 야생식물로서, 아직가사화와 육종, 그리고 약학적 이용성에 대한 연구가 많이 수행되지 않았다. 최근에 이르러서야 전사체 및 대사체 분석을 통한 데이터의 양산으로 유전적, 육종적 그리고 약학에 이르는 연구를 가능하게 만들었다. 재배종으로의 개발과 고들빼기 육종 향상을 위해서는 야생종의 유전적 다양성을 조사하는 것이 중요한 의미를 갖는다. 더 나아가 이를 위해 기내 증식 실험 기반의 확보는 상업적인 재배와 다양한 생물학적 이용에 기여 할 수 있다.

이에 본 연구는 먼저 왕고들빼기 전사체 염기서열 데이터로부터 유전자영역에 있는 단순반복염기서열(simple sequence repeat [SSR]) 표지들을 개발하여 유전적 다양성을 연구하며 마커의 양도성을 연구하였고, 유전적 다양성과 농업적인 특성을 분석하기 위해 형태학적 특성들을 조사 하였다.새로이 개발된 SSR 표지들은 높은 다형성을 나타내었고, 유전자 좌당 10.83 대립형질을 보였고, 평균 기대이형접합성율은 0.76 으로 관찰치(0.39)보다 높았고 변이의 대다수는 지역간 보다 지역내 개체간에 존재하였다. 이는 높은 유전적 다양성을 의미한다. 집단구조, 계통도 그리고 주성분 분석에서 *L. indica* 수집종들은 약간의 혼잡이 있지만 북부와 남중부 두 개 집단으로 나뉘어 졌고 몇몇 수집종들은 *L. sativa* 와 *L. serriola* 와 유전적 관련성을 보였다.

한편, 73 개 수집종들중 38 개 수집종들에 대해 농업 형태학적 특성을 조사하고 이것들에 대한 다양성을 분석하였다. 모든 형질에 대해 유전적 평균분산제곱은 유의미하였고 ($P<0.001$) 이는 수집종들간 높은 변이를 의미한다. 모든 형질들에서 높은 광의의 유전력(>60%)을 나타내었다. 높은 백분율평균으로서의 유전적 진전(genetic advance as percentage mean [GAM])을 보인 형질들(엽장, 엽폭, 분지수, 마디수, 초장, 기부와 중간 마디 간격, 천립중 등)은 상가적 유전자 효과(additive gene effect)을 보이고 이는 표현형적 관찰을 토대로 한 선발은 이러한 형질들의 보다 좋은 개선을 위해 효과적임을 의미한다. 중간정도의 GAM 을 보이는 형질들(추대기간, 개화기, 결실률, 종자성숙 등)은 교잡과 선발을 통해 생산성 향상에 기여할 수 있다. 수집종 8 번과 55 번은 우수한 농업 형태적 특성을 보이므로 추후 연구 및 육종을 위한 좋은 재료가 될 수 있다.

본 연구는 또한 왕고들빼기의 조직배양을 통한 효율적인 육종을 위하여 기내 증식 방안에 대한 연구를 수행하였다. 앞선 연구에서 우수한 농업 형태적 특성을 보이는 왕고들빼기 수집종 55 번으로부터 잎과 엽병을 재료로 사용하여 Murashige & Skoog (MS) 배지를 기본으로 하여 다양한 농도의 6-benzylaminopurine (BAP)와 indole-3 acetic acid (IAA)를 첨가하여 조직배양을 수행하였다. 0.5 mg L^{-1} BAP 와 1.2 mg L^{-1} IAA 를 첨가한 MS 배지에서 신초생성이 가장 좋았고, 4 mg L^{-1} BAP 와 1.2 mg L^{-1} IAA 를 첨가한 MS 배지에서 캘러스 유도가 가장 좋게 나타났다. 1.5 mg L^{-1} BAP 와 0.5 mg L^{-1} IAA 를 첨가한

배지에서 캘러스 재생이 가장 우수하였고 기내발근유도에 가장 효과적인 호르몬 농도는 1 mg L^{-1} IAA 이었다. 또한 식물의학적 연구에서 조직배양 적용성을 알아보기 위해 실외 그리고 기내에서 자란 조직들로부터 lactucin 을 함량을 비교하고 항세균성을 조사하였다. 모든 조직들의 추출물에서 lactucin 이 탐색되었고 한천절편확산(agar disc diffusion) 방법을 통해 *Pseudomonas fuscovaginae* (벼 병균)과 *Escheriea coli* 에 대한 항세균성을 확인하였다.

본 연구에서는 왕고들빼기 전사체 염기서열로부터 SSR 마커를 개발하고 한국 왕고들빼기 수집종 집단에 대해 SSR 마커와 여러 농업 형태적 형질들을 조사하여 유전적 다양성을 분석함으로써 왕고들빼기의 상업적 재배를 위한 육종 프로그램에서 선발 도구와 유용한 재료로 사용될 수 있을 것이다. 또한 본 식물의 보존과 식물의학적 활용을 위한 기내 증식 체계를 확립하였다.

중심어: 왕고들빼기(*Lactuca indica* L.), SSR 마커, 유전적 다양성, 농업적 특성, 기내 증식, 락투신, 항세균성 분석

학번: 2015-30859

ACKNOWLEDGEMENT`

I would like to express my sincere gratitude to my advisor Prof. Suk-Ha Lee for providing me the opportunity to do my Ph. D study and related research in his research lab at SNU with his constant support, guidance, motivation, and encouragement. I am one of the most fortunate candidate to have a wonderful academic advisor like him. My sincere gratitude also goes to my defense committee members: Prof. Byeon Woo Lee, Prof. Hak Soo Seo, Prof. Do-Soon Kim, and Dr. Jae Gyun Gwag, for letting my defense be an enjoyable moment, and for their brilliant comments and suggestions.

I would specially like to thanks to Dr. Moon Young Kim, for her constant guidance, insightful comments and suggestion for my entire research work and helping me out with manuscript preparation and submission, Dr. Jung Min Ha whose insightful comments and suggestion not only helped me to improve this dissertation but also helped for defense preparation, Dr. Min Young Yoon for her incredible help for academic and non-academic matters, and making life in SNU and Korea more comfortable. My gratitude also goes to Dr. Yeong-Ho Lee, Dr. Keum Yong Park and his staff at Suwon field for helping me to make my experimental work smooth.

I am also grateful to current researcher and lab members of Crop Genomics Lab: Hyun Ju Jang, Sangrea Shim, Taeyoung Lee, Jayern Lee, Xuefei Yang, Eunsoo Lee, John Bwalya, Jun Hee Jung, Eum Sam Park, Eunbi Jo, Hakyung Kwon and Kang-Heum Cho for providing their untiring support, care and love for achieving this success.

I would like to remember the alumni from the lab: Dr. Dani Satyawan, Haneul Jeong, Andari Risiawati and Tantri Anggraeni for their support. I am also thankful to Dr. Kularb Laosatit for encouraging me and helping with my research work during her staying in Korea.

I would like to express my healthful gratitude to my beloved parents, father Mr. Krishna Raj Oliya and mother Mrs. Laxmi Devi Oliya for seeding the dream of Ph. D to me from the very early stage of my academic carrier to till now with their unlimited love, constant support, motivation, and courage.

I am also blessed with most intelligence, helpful and lovely husband Mr. Bikram Acharya. His great support, understanding, encouragement, and guidance which I got both in academic and household problems during my pursuit of Ph. D degree, that made the accomplishment of this thesis possible. He was always around at times when I was in problem and helped me to keep things in perspectives. I greatly value his contribution and deeply appreciate his believe on me. I am also feeling lucky to be blessed by two cute and beloved daughters:

Shilisa, for being such a good girl for abiding my ignorance and always cheering me up and Salvia, for arriving in my life as a catalyst during my Ph. D work and for her cute smile and the patience she showed during my thesis writing, I would like to dedicate this achievement to my beloved family.

My heartfelt regards go to my father-in-law and mother-in-law, for their moral support. I am also grateful to all my family members and relatives for being with me in every difficulty. I am also thankful to my seniors from Nepal, specially Dr. Shanti Devi Upadhaya and Dr. Puna Maya Maharjan not only for their encouragement and suggestions during my Ph.D. but also for their great help for taking care of our babies when we were in need. Last but not the list, I would like to express my grateful thanks to Seoul National University for providing GSFS scholarship for my tuition fee and stipend for the prescribed period. Thank you God for all your blessings to me and my family.