African Journal of Biotechnology Vol. 8 (15), pp. 3440-3447, 4 August, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Habitat fragmentation causes rapid genetic differentiation and homogenization in natural plant populations – A case study in *Leymus chinensis*

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Accepted 5 June, 2009

The effects of habitat fragmentations on the forage grass *Leymus thinness* (Trin.) Tzvel, which has high genetic diversity in northeast China were investigated. Four natural populations of the same ecotype (Grey-green leaf, GGL), namely, BT, ZL, CL and CC (named after location) were collected from different abiotic growing conditions. The CC population has become isolated in a park inside a city by tall buildings though geologically close to CL. Amplified fragment length polymorphism (AFLP) selected primer combinations were highly efficient in revealing the inter-clonal and inter-populational genetic variation in this species. The genetic diversity indices were higher in BT (H = 0.2305) and ZL (0.2467) populations and the lowest in CC (0.1674) population. Cluster analysis showed that the CC population was becoming isolated from the rest with the least gene flow from BT (1.51) as compared from BT to ZL (2.24). Lowest polymorphism was observed in CC (52.31%) as compared to CL (57.69%), BT (70.00%) and ZL (70.38%); this showed a tendency towards homogenization probably due to increased selfing, and due to reduced gene flow apparently caused by city buildings. These results were supported by multiple statistical analyses including Mantel's test, PCOORDA and AMOVA. Genetic enrichment and epigenetic variation studies can be included in habitat fragmentation analysis and its implications in inducing homogenization and susceptibility in natural plant populations.

Key words*: Leymus chinensis,* AFLP, genetic diversity, habitat fragmentation.

INTRODUCTION

One of the broadly distributed perennial and rhizome propagating species in the Songnen plain in the northeast of China is *Leymus chinensis* (Trin.) Tzvel. [Syn. *Aneurolepidium chinensis* (Trin.) Kitag]. It is a constructive dominant species which grows across diverse soils and climatic conditions (Jia, 1987; Zhou and Yang, 2003a). The Songnen plain is a grazing field and has been experiencing an impact of human population and climatic changes at both local and global scales. This has caused severe soil degradation and vegetation deterioration. Hence there is a daring need of ecological renovation to sustain the former ecosystem dynamism in the area. The *L. chinensis* holds a special promise as a suitable plant species candidate for this ecological renovation hence a timely understanding and utilization of its genetic variability and adaptability can be used as a stepping stone in identifying germplasms that can be used in the search of new ecotypes with novel genes which can also be incorporated in crop, fodder and cover tree improvement programs in other similar reclaimable ecological zones. A considerable genetic variability within or between natural populations provides a genomic flexibility that can used as a raw material for plant adaptation as pioneer species. This is because low genetic variability often reduces the capacity to adapt to changing environmental conditions (Ellstrand and Elam, 1993) and hence an inability to cope with abiotic and biotic stresses (Valen, 1965). The pattern and spectrum of genetic variations within or between plant populations can be compared and analyzed using molecular genetic tools (Madan et al., 2002; Reisch et al., 2005). One of the most precise, cost effective polymerase

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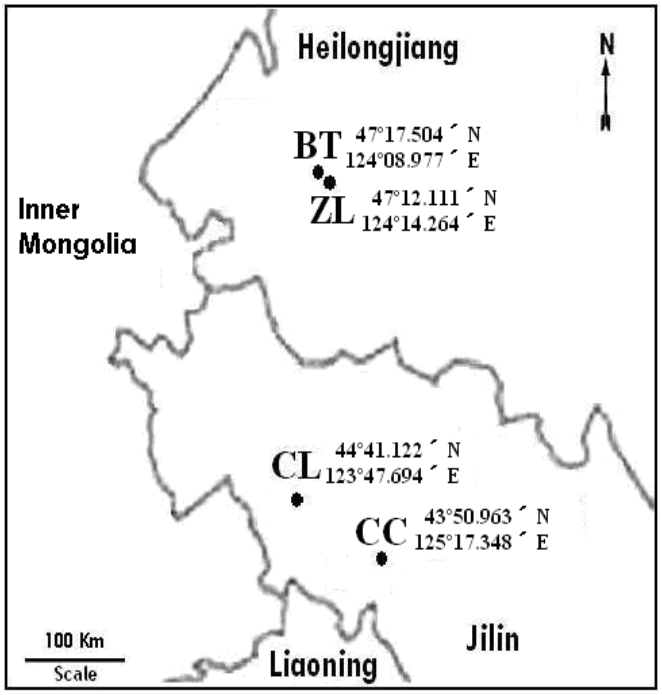

Figure 1. Distribution of the geographic localities of the four *L. chinensis* natural populations across the Songnen Plain in Northeast of China., the specific coordinates of each collection site is indicated.

chain reaction (PCR) based ecological tool for molecular analysis of such population genetic diversity analysis is the AFLP (amplified fragment length polymorphism) (Vos et al., 1995; Bensch, and Akesson, 2005) since it has been highly informative, reliable and efficient tool in other grass investigations (Fjellheim and Rognli, 2005). Earlier studies using *random amplification of polymorphic DNA* (RAPD) and AFLP molecular markers showed relatively high genetic variations and differentiations among the populations in the area, and a significantly positive correlation between genetic differentiation and geographic distance among the populations. The level of genetic differentiation was most likely affected by more than one ecological factor, and the genetic variation in a small geographic region was determined by the environmental heterogeneity (Liu et al., 2004). Natural mutation, artificial selection etc, combined together with ecological factors could have made *L. Chinensis* to differentiate into various ecotypes (Liu et al., 2002). Self-incompatibility in this species contributes to evident genetic differentiation together with the environment habitat selection pressure coupled with low levels of gene flow (Gong et al., 2007). Its habitat revegetation is influenced by artificial selection, habitat selection pressure, environmental heterogeneity, genetic differentiation and diversity which are related to the wind-mediated dispersal of pollen and seed transport via migrating birds (Liang et al., 2007).

The natural populations of *L. chinensis* in the Songnen Plain have evolved two easily recognizable phenotypes which are hereby indicated as; grey-green leaf (GGL) and yellow-green leaf (YGL). These have adapted to contrasting ecological habitats with the GGL type often thriving in eroded and highly stressful alkaline/saline meadows of pH 8.5 to 9.5) while the YGL grows only in normal edaphic conditions of pH < 8.0 (Zhou and Yang, 2003b). In this study, AFLP data of four populations of the same ecotype (GGL) indicated as BT, ZL, CL and CC were analyzed. The CC ecotype which was found in the south of the Songnen plain in a park inside the city of Changchun and surrounded by tall buildings besides a man-made lake, was found to have differentiated separately from the other ecotypes. The ZL and CL grew in saline/alkaline (pH 8.5 to 9.5) conditions and Cluster analysis grouped them together indicating that some genetic or epigenetic changes could have been triggered by the abiotic stress along side the other barriers. The analysis also showed that physical or biological distances had a key role in determining the level of gene flow between ecotypes and that habitat fragmentation causes genetic differentiation and homogenization in a species.

MATERIALS AND METHODS

Plant materials

Distinct forty eight clones (twelve clones from each population) of *L. chinensis* belonging to the same ecotype (grey-green leaf, GGL) were randomly selected from four different natural populations, which included CC, BT, ZL, and CC (named after locality) and distributed at the certain geographic range with different degrees of the soil pH values in the Songnen Plain in northeast China (Figure 1). Two populations, CC and BT, were growing in normal soil with lower average soil pH values about 7.0, although the CC was growing in the centre of the city surrounded by tall buildings. The two populations, ZL and CL were growing respectively in alkaline/saline wet land and meadows with higher average soil pH values ranging from 8.5 to 9.5.

DNA isolation and AFLP amplification

Growing young green expanded leaves were taken from the selected clones for DNA extraction. Genomic DNA was isolated by a modified CTAB method (Kidwell and Osborn, 1992), and purified by phenol extractions. The standard AFLP (amplified fragment length polymorphism) analysis (Vos et al., 1995) was performed with minor modifications (Liu et al., 2007). The primers used and their sequence information are as provided in Supplementary Tables 1 and 2. Silver stained sequencing gel was used to resolve and visualize the AFLP amplification products. Two sets of independent AFLP analyses were performed for all samples to ensure reproducibility of scored bands.

Band scoring and data analysis

The band scoring and data analysis were carried out following the same criterion and methods in the AFLP. Only well-resolved and reproducible bands at a given locus on the AFLP gel profile of each primer were scored and transformed into a binary character matrix,

Table 1. AFLP adapters and pre-selective primers used in this study.

Name	Sequence
Mse I-adaptor I	5'-GACGATGAGTCCTGAG-3'
Mse I- adaptor II	5'-TACTCAGGACTCAT-3'
EcoR I- adaptor I	5'-CTCGTAGACTGCGTACC-3'
EcoR I- adaptor II	5'-AATTGGTACGCAGTC-3'
Pre-amplification primer	
EcoR I+A	5'-GACTGCGTACCAATTCA-3'
Mse I+C	5'-GATGAGTCCTGAGTAAC-3'

Table 2. Selective AFLP amplification primer pairs used in this study.

Primer pairs	*Eco*RI primers	*Mse* I primers
1b	5´- GACTGCGTACCAATTC**AAG**-3´	5´- GATGAGTCCTGAGTAA**CAA**-3´
1f	5´- GACTGCGTACCAATTC**ACG**-3´	5´- GATGAGTCCTGAGTAA**CAA**-3´
1g	5´- GACTGCGTACCAATTC**AGC**-3´	5´- GATGAGTCCTGAGTAA**CAA**-3´
1h	5´- GACTGCGTACCAATTC**AGG**-3´	5´- GATGAGTCCTGAGTAA**CAA**-3´
6b	5´- GACTGCGTACCAATTC**AAG**-3´	5´- GATGAGTCCTGAGTAA**CTC**-3´
6d	5´- GACTGCGTACCAATTC**ACT**-3´	5´- GATGAGTCCTGAGTAA**CTC**-3´
6f	5´- GACTGCGTACCAATTC**ACG**-3´	5´- GATGAGTCCTGAGTAA**CTC**-3´
6h	5´- GACTGCGTACCAATTC**AGG**-3´	5´- GATGAGTCCTGAGTAA**CTC**-3´
6i	5´- GACTGCGTACCAATTC**AGA**-3´	5´- GATGAGTCCTGAGTAA**CTC**-3´
7a	5´- GACTGCGTACCAATTC**AAC**-3´	5´- GATGAGTCCTGAGTAA**CTG**-3´
7b	5´- GACTGCGTACCAATTC**AAG**-3´	5´- GATGAGTCCTGAGTAA**CTG**-3´
7f	5´- GACTGCGTACCAATTC**ACG**-3´	5´- GATGAGTCCTGAGTAA**CTG**-3´
8a	5´- GACTGCGTACCAATTC**AAC**-3´	5´- GATGAGTCCTGAGTAA**CTT**-3´
8c	5´- GACTGCGTACCAATTC**ACC**-3´	5´- GATGAGTCCTGAGTAA**CTT**-3´
8e	5´- GACTGCGTACCAATTC**ACC**-3´	5´- GATGAGTCCTGAGTAA**CTT**-3´
8g	5´- GACTGCGTACCAATTC**AGC**-3´	5´- GATGAGTCCTGAGTAA**CTT**-3´
8h	5´- GACTGCGTACCAATTC**AGG**-3´	5´- GATGAGTCCTGAGTAA**CTT**-3´
8j	5´- GACTGCGTACCAATTC**ATC**-3´	5´- GATGAGTCCTGAGTAA**CTT**-3´

where “1” stands for presence and “0” for absence at a given position in the marker profile. Genetic similarity between pairs of clones was estimated by the Jaccard index, which was considered appropriate for dominant markers like AFLP, as it does not attribute any genetic meaning to the coincidence of band absence (Mattioni et al., 2002). The similarities were calculated by the formula:

$$\text{Jaccard index} = N_{AB} / (N_{AB} + N_A + N_B).$$

Where N_{AB} is the number of bands shared by two samples, N_A represents bands only in sample A and N_B represents bands only in sample B. The dendrogram was generated by cluster analysis based upon the unweighted pair group method with arithmetical averages (UPGMA), which compresses the patterns of variation into two-dimensional branch diagrams (Sneath and Sokal, 1973) in the NTSYS-pc (Numerical Taxonomy System pc) version 2.0 package (Rohlf, 1998). The robustness of the dendrogram was tested by estimating the cophenetic correlation values, and comparing them with the original similarity matrix using Mantel’s matrix correspondence test (Mantel, 1967) based on 1000 random permutations. Principal coordinate analysis (PCOORDA) was also performed in the NTSYS-pc version 2.0 package to ordinate relationships between clones and populations, which highlights the resolving power of the ordination (Mattioni et al., 2002). The genetic diversity indices of each population were calculated by the Popgene software package, version 1.21 (Yeh et al., 1997), and the details about Gene frequency, Percentage of polymorphic loci (*P*) (Nei et al., 1995), Nei’s gene diversity index (*H*) (Nei, 1973), and Shannon information index (*I*) (Lewontin, 1972) were referred (Gong et al., 2007; Li et al., 2009).

To test the genetic diversity within the four populations, between populations within the same artificially divided group, and among clones within each of the populations, the non-parametric analysis of molecular variance (AMOVA) (Excoffier et al., 1992) using Arlequin version 3.01 (Excoffier et al., 2005) was applied. AMOVA was based on the pairwise squared Euclidian distances between molecular phenotypes, which are equal to the number of different band states, because band states can only take the values “0” or “1” (Reisch et al., 2005). It allowed the calculation of variance components (the divided groups, four populations within the groups and clones within each of the four populations) and their significance levels for variation. The occurrence of gene flow (N_m) between the

Table 3. Summary of the information indices of the selected AFLP primer pairs.

PP	NB	MB	PB	PPB (%)	PIC
1/b	46	36	10	21.73	0.3403
1/f	48	32	16	33.33	0.4444
1/g	54	43	11	20.37	0.3244
1/h	83	53	30	36.14	0.4616
6/b	45	38	7	15.55	0.2627
6/d	80	61	19	23.75	0.3622
6/f	36	29	7	19.44	0.3133
6/h	44	34	10	22.73	0.3512
6/i	56	40	16	28.57	0.4082
7/a	47	38	9	19.15	0.3096
7/b	65	40	25	38.46	0.4734
7/f	81	52	29	35.80	0.4597
8/a	72	57	15	20.83	0.3299
8/c	37	32	5	13.51	0.2337
8/e	66	49	17	25.76	0.3825
8/g	40	31	9	22.50	0.3488
8/h	44	36	8	18.18	0.2975
8/j	77	60	17	22.08	0.3441
Total	1021	761	260		
Mean	56.72	42.27	14.44	25.47	0.3582

PP: primer pairs, NB: number of bands, MB: number of monomorphic bands, PB: number of polymorphic bands, PPB: percentage of polymorphic bands, PIC: polymorphic information content. PIC=2*f (1-f), where f is the percentage of the polymorphic bands out of the total bands.

populations was estimated using the equation:

N_m = ¼ (1/G_{st} -1).

Where G_{st} is the pairwise genetic distance between populations calculated by the above AMOVA algorithm (Wright, 1978). The dendrograms of Nei's unbiased measures of genetic distance (Nei, 1978) AFLP based on using UPGMA was employed to directly increase the demonstration of relationship between the four populations (Nei, 1987).

RESULTS

Evaluation of AFLP primer combinations

Based on a comparison of the amplification profiles between duplicates of DNA from a single *L. chinensis* clone, 18 most suitable AFLP primer combinations were selected out of 90 tested primer pairs (Tables 1, 2, 3 and Figure 2); 1021 AFLP bands were scored based on complete reproducibility between two independent amplifycations from each of the 48 clones (that is, the whole experiment was performed twice) taken from the four populations, CC, BT, ZL, and CL; of the bands scored, 260 were found to be polymorphic among the clones. The indices of the 18 primer pairs were computed and shown in Table 3, which included the number of monomorphic and polymorphic bands (MB and PB), percentage of polymorphic bands (PPB) and polymorphic information content (PIC). Apparently, all 18 selected primer pairs are informative in detecting genetic variations in *L. chinensis*, albeit with different efficiencies. For example, primer pair '7b' generated the highest percent of polymorphic bands (38.46%) and the highest values for PIC (0.4734), whereas '8c' generated the lowest values for these indices. The average values for each of the indices for the 18 selected primers, NB, MB, PB, PPB and PIC, were 56.72, 42.27, 14.44, 25.47 and 0.3582% respectively (Table 3).

Cluster analysis

The UPGMA clustering algorithm grouped the 48 clones into four distinct clusters (Figure 3) in accordance with their population origins and was related to their abiotic conditions. The associations between the original genetic similarity matrix and the cophenetic correlation matrix were found to be highly significant based on Mantel's test ($r = 0.908$, $p = 0.002 < 0.01$), thus testifying authenticity of the dendrograms. The relationships among the *L. chinensis* clones and among the populations were displayed by PCOORDA based on the AFLP data (Figure 4). In the two-dimensional plot, dimension-1 (accounting for 15.98% of the variation) clearly depicted the population-level genetic differentiation among the four populations, which corresponded to their localities, except for ZL and CL, whereas dimension-2 (accounting for 11.35% of the variation) pointed to separation of the ZL and CL populations.

Genetic diversity and gene flow within and among populations

Based on the AFLP data, the polymorphic levels within each of the four populations was lowest in CC (52.31%) and highest in BT (70.38%) and ZL (70.00%) as shown in Table 4. The Nei's gene diversity indices (Nei, 1978) of ZL (0.2467) and BT (0.2305) were higher than those of CL (0.1768) and CC (0.1674). The Shannon information indices (Lewontin, 1972) of ZL (0.3674) and BT (0.3484) were also higher than those of CL (0.2709) and CC (0.2544). Hence, the three genetic diversity indices showed that the two populations of ZL and BT harbored a higher level of genetic diversity than the other two populations of CL and CC.

The occurrence and spectra of gene flow through either seed and/or pollen dispersal would have imposed a significant impact on intra- and inter-population genetic variations. The gene flow value (N_m) among the four populations was 0.93, the N_m between CC and CL (0.89) was lower than those of the other population pairs, which may be brought by some isolation factors between CC and

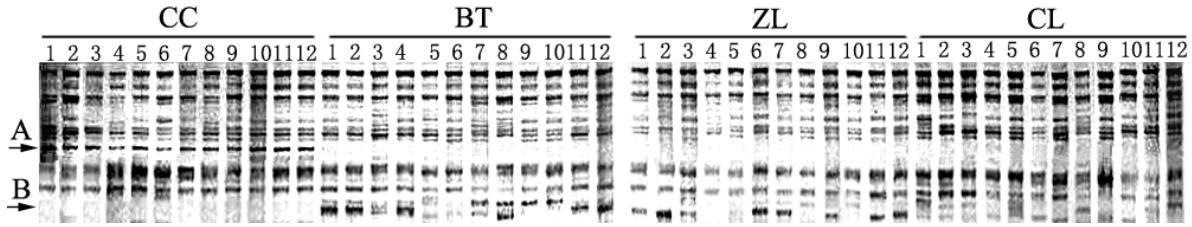

Figure 2. Examples of the population-specific (marked by an arrowhead prefixed with A) and population-enriched (marked by an arrowhead prefixed with B) bands identified in the AFLP profiles of *L. chinensis* clonels. A and B were portions of AFLP profiles generated by primer pairs '7f'.

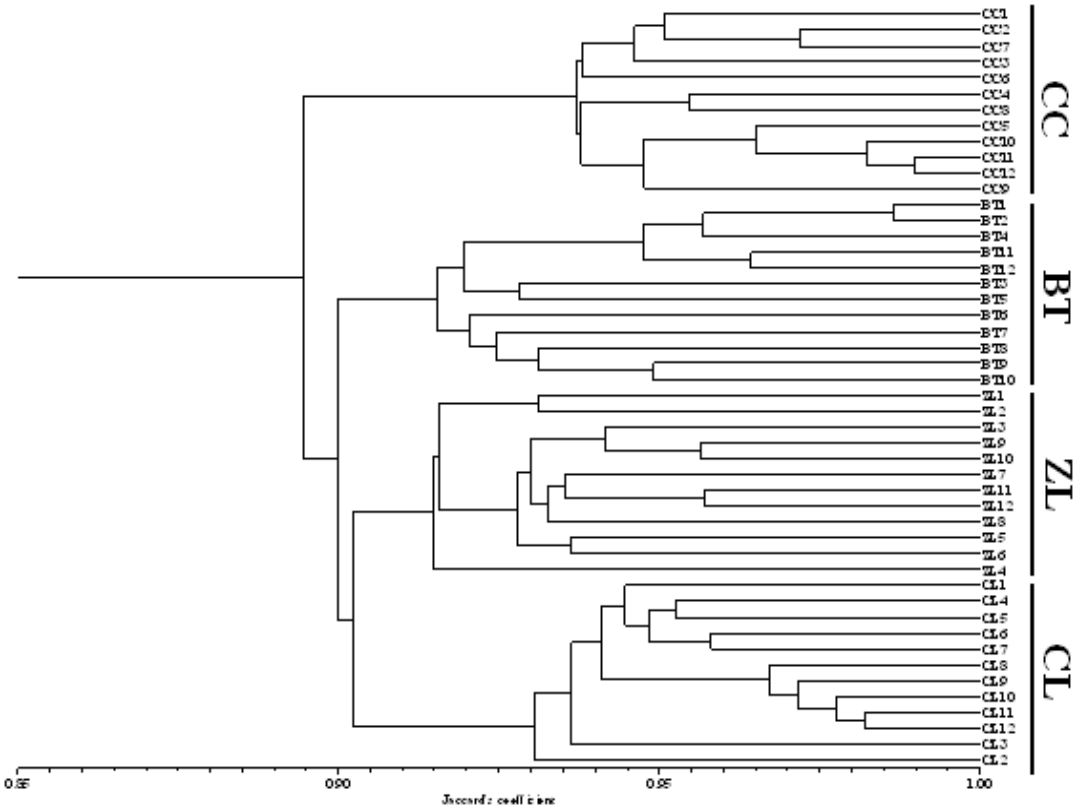

Figure 3. Dendrogram of *L. chinensis* populations constructed using the UPGMA method (Sneath and Sokal 1973), based on a similarity matrix of AFLP data calculated according to the Jaccard index. 48 clones are clustered into four distinct groups, CC, BT, ZL, and CL in line with their population origins.

CL. As expected, N_m between ZL and BT was the largest (2.24), which is most likely because of their proximity in geographical distance. The gene flow frequencies between rest population pairs were correlated with the geographic distances (Table 5 and Figure 1).

AMOVA

The AMOVA program was used to partition the genetic variation by hierarchical analysis from the distance matrix. An AMOVA revealed 36.65% of the genetic variation among populations, and 63.35% within the population (Table 6). The upper results, together with the PCOORDA (Figure 4) and the Dendrograms of Nei's unbiased measures of genetic distance (Table 5) (Nei, 1978) based upon AFLP markers using unweighted pair group method with arithmetical averages (UPGMA) (Nei, 1987), indicated clear inter-populational differentiation, which has led to isolation of the populations of ZL and BT. The group-level AMOVA, revealed 26.17% of the genetic variation between, and 62.11% within the populations respectively (Table 6). The AMOVA also showed a high and significant ($p < 0.001$) genetic differences between the BT and ZL,

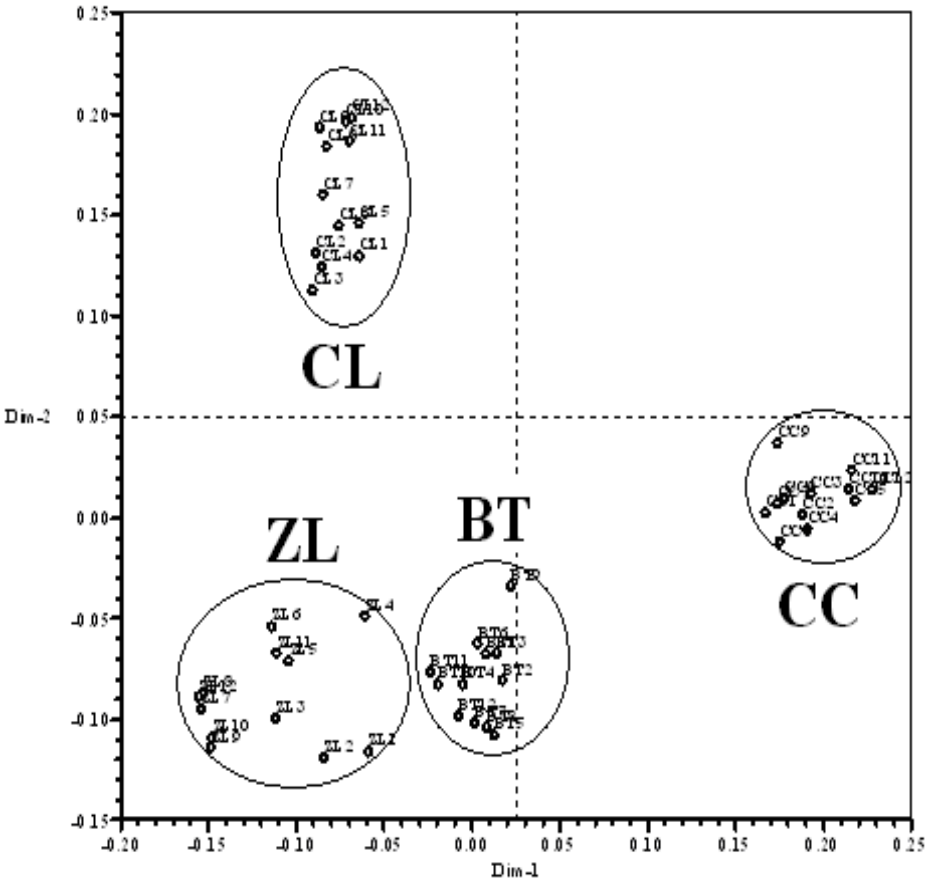

Figure 4. Principal coordinate analysis (PCOORDA) of the 48 *L. chinensis* clones. The two-dimensional results of PCOORDA based on the AFLP data. Axis dimensional-1 extracted 15.98% of the variance and axis-dimensional-2 extracted 11.35% of the variance. The two-dimensional ordination has clearly separated the clones into four groups according to their population origins.

Table 4. Genetic diversity within the four populations of *L. chinensis* based on 260 polymorphic AFLP bands (*P*: percentage of polymorphic loci, *H*: Nei's gene diversity index, *I*: Shannon information index. The values in parentheses are percentages for each of the indices.).

Population	*P* (%)	*H*	*I*
CC	52.31	0.1674 (0.1917)	0.2544 (0.2754)
BT	70.38	0.2305 (0.1934)	0.3484 (0.2714)
ZL	70.00	0.2467 (0.2008)	0.3674 (0.2807)
CL	57.69	0.1768 (0.1893)	0.2709 (0.2709)
Mean	**62.60**	**0.2054 (0.1938)**	**0.3103 (0.2746)**

Table 5. Gene flow values (*Nm*, left below diagonal) and Nei's genetic distance (Nei, 1978, right above diagonal) between pairs of the four *L. chinensis* populations based on the 260 polymorphic AFLP bands.

Population	CC	BT	ZL	CL
CC		0.1678	0.2390	0.2582
BT	1.51		0.1372	0.1906
ZL	1.17	2.24		0.1705
CL	0.89	1.40	1.60	

the CL group and the CC group and between clones within each of the populations (Table 6). The inter-population relationships were also more straightforwardly depicted by the dendrograms of Nei's unbiased measures of genetic distance (Nei, 1978) based upon AFLP markers using unweighted pair group method with arithmetical averages (UPGMA) (Nei, 1987) (Figure 3).

DISCUSSION

The AFLP data revealed and confirmed earlier high levels of inter-clonal genetic diversity within each of the populations of *L. chinensis* with the same ecotype which has the

Table 6. Summary of analysis of molecular variance (AMOVA) based on the 260 polymorphic AFLP bands (two-level-AMOVA). (SS: sums of squares, V.P.: variance components, PGV: proportion of genetic variability, *P*: level of significance, levels of significance test were based on 1000 permutations.)

Level of variation	SS	V.P.	PGV (%)	*P*
Without population grouping				
Among populations	721.354	17.5147	36.65	< 0.001
Within population	1332.083	30.2746	63.35	< 0.001
Population groups based on location (CC, CL, BT and ZL)				
Between groups	538.021	5.7118	11.72	< 0.001
Between populations within groups	183.333	12.7548	26.17	< 0.001
Within population	1332.083	30.2746	62.11	< 0.001

green grey leaf (GGL). This was reflected by the variable ranges of the genetic diversity indices including percentage of polymorphic loci (*P*), Nei's genetic diversity index (*H*) and Shannon information index (*I*), for each of the markers. The clustering, two-dimensional PCOORDA analysis on data of both markers and assignment test on the AFLP data, both of which were further supported by AMOVA, clearly pointed to the existence of substantial inter-population genetic differentiation. There was a remarkable observation in the analysis which showed that the soil condition and the proximal distance between the populations had a significant influence in the genetic diversity analysis. It is conceivable that the occurrence and spectra of genetic variations, both within a plant population and among populations, are mainly caused by three distinct as well as interlaced factors, that is, gene flow through seed dispersal and/or pollen dissemination, genetic drift and habitat induction. Of the first two factors, when the levels of gene flow are low, then genetic drift becomes the predominant factor in shaping the population genetic structure and results in within-population homogeneity but among population differentiation (Reisch et al., 2005). With regard to gene flow, for a given plant species, although the most easily conceivable scenario is causal links with geographic distance, in the present study; however, it is noted that under certain circumstances, that is, the frequency between CL and CC populations pair showed little relatedness to geographic distances between CL and CC (Table 5). It can be envisioned that a multiple other factors might have imposed their influences on the efficiency or effectiveness of gene flow among the *L. chinensis* populations. First, the Songnen Prairie as a whole is a flat plain and without barriers like mountains. Hence gene flow might be easy as shown in BT and ZL which had a high gene flow. The low gene flow between them and CL and CC could be due to the distance and other shielding vegetations, accumulation of valleys which could influence the flow of wind and movement of dispersal agents. The CC population was seen as being isolated due to its special predicament of its locality. Being in the midst of tall buildings seems to have raised a great barrier which is gradually isolating it from the rest. Other biologically influenced dispersal agents might have been hindered from enriching this clone with new variations from outside; which could interfere with wind and pollinators movements (Li et al., 2009). Hence homogenization is being gradually established in this clone due to the habitat fragmentations caused by human activities. The exceptionally low level of gene flow between CL and CC populations (Table 5) provided a typical example to suggest that genetic isolation is not necessarily determined by distance, but other factors could also have a role in the differentiation of these populations, although, physical and/or biological isolation seem to be the most significant. But although the soil pH values as a habitat heterogeneity were not correlated with genetic variation intrapopulation of *L. chinensis* with the same ecotype, it is interesting to note that the dendrogram of *L. chinensis* populations constructed by the UPGMA method (Sneath and Sokal, 1973), based on a similarity matrix of AFLP data calculated according to the Jaccard index showed a close link between the clones grown in the same abiotic conditions (Figure 3). Like CC was more closely related to BT (all grown at pH 7) and CL more closely linked to ZL which are grown in soils of pH 8.5-9.5. The possibility of habitat inducement can not be ruled out in causing heterogeneity in stressful conditions of varied salinity/alkaline which have been known to cause heritable epigenetic variations in plants.

ACKNOWLEDGEMENT

This study was supported by the National Natural Science Foundation of China (30870178).

Supplementary materials

AFLP adapters, pre-selective primers, selective AFLP amplification primer pairs, summary of the information indices of the selected AFLP primer pairs used in this study, and examples of the population-specific bands generated by primer pairs '7f' as identified in the AFLP profiles of *L. chinensis* clones.

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