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Full Length Research Paper

Phylogenetic relationships of eleven *Kobresia* accessions from the Tibetan plateau

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In the past, identification of the genus *Kobresia* was mostly dependent on morphological characteristics. This study used random amplified polymorphic DNA (RAPD), sequences of nrDNA ITS, cpDNA trnT-L-F spacer and cpDNA ndhF to assess the phylogenetic relationships among the accessions of *Kobresia* plants collected from the Tibetan plateau. In the dendrograms, *Kobresia macrentha* (L) species formed a separate clade suggesting a remote relationship with other accessions. These trees showed that species found in similar habitats or having similar adaptations tended to cluster together. Thus, the genetic variation and adaptation seen in these *Kobresia* accessions may be due to their remote geographic and high altitudinal position in the Tibetan plateau. This study highlights the importance of molecular analysis in understanding the genetic diversity and structure of *Kobresia* accessions, and contributes to the knowledge of conservation of genetic resources.

Key words: *Kobresia*, phylogenetic relationships, random amplified polymorphic DNA, Tibetan plateau.

INTRODUCTION

The Tibetan plateau is the highest plateau in the world and is often called 'the third pole'. It is an important part of the global terrestrial ecosystem on the Eurasian continent and a key region for flora genetic resources that influence ecosystems and evolutionary processes (Chang, 1983). Two third of the plateau area, about 1.5 million km^2, is grassland (Sun and Zheng, 1998). The native alpine steppe meadow is characterized by the dominance of sedges and grasses (Zhou and Deng, 2001). *Kobresia* species from sedges are perennial herbs and are the primary types of vegetation distributed in the Tibetan plateau. These species not only have the good nutritional quality preferred for livestock, but also maintain important ecological functions in the regional ecosystems. The genus *Kobresia* Willd. belongs to the tribe Cariceae in the family Cyperaceae. This genus has about 70 species all over the world, of which 59 were found in China (Zhou and Deng 2001). The genus conserves water resources, regulates river systems and regional climates and supports regional biodiversity.

The primary threats to *Kobresia*'s habitat on the Tibetan plateau are hydrologic alterations, overgrazing and sod removal for construction. These have resulted in vast areas of "black soil" and have been correlated with global climate change (Sun and Zheng, 1998). In addition, lack of information on the species' population genetics, adaptation and dispersal biology adds to the potential severity of impact on *Kobresia* by these threats. This study addresses phylogenetic relations of 11 *Kobresia* accessions collected in this region in order to have a better understanding of the genetic diversity and provide information necessary for appropriate management of these plants in the future.

Diversity of *Kobresia* species has been studied in relatively few areas of the Tibetan plateau because of its

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Abbreviations: RAPD, Random amplified polymorpfhic DNA; **AFLP,** amplified fragment length polymorphism; **GIS,** geographic information system; **CTAB,** cetyl trimethy-lammonium bromide; **PCR,** polymerase chain reaction; **PIC,** polymorphism information content; **ITS,** internal transcribed spacers; **PPB,** percentage of polymorphic bands.

Table 1. Populations of *Kobresia* from Tibetan plateau of China in this study.

Species	Origin	Altitude (m)	Longitude and latitude	Habitat
K. royleana (N) *B.*	Langkazi	4455	28°59′11N 90°26′04E	Alpine steppe
K. macrentha Boeck.	Langkazi	4455	28°59′11N 90°26′04E	Alpine meadow
K. littledalei C. B. Clarke	Langkazi	4455	28°59′11N 90°26′04E	Alpine meadow
K. royleana (N) *B*	Naqu	4456	31°26′34N 92°16′32E	Alpine steppe
K. prattii C. B. Clarke	Dangxiong	4278	30°29′35N 91°05′58E	Alpine steppe
K. caillifolia Decne	Dangxiong	4278	30°29′35N 91°05′58E	Alpine meadow
K. littledalei C. B. Clarke	Naqu	4456	31°26′34N 92°16′32E	Marshy meadow
K. littledalei C. B. Clarke	Dangxiong	4278	30°29′35N 91°05′58E	Marshy meadow
K. humilis C. A. Mey	Naqu	4456	31°26′34N 92°16′32E	Alpine steppe
K. humilis C. A. Mey	Dangxiong	4278	30°29′35N 91°05′58E	Alpine steppe
K. pygmaea C. B. Clarke	Naqu	4456	31°26′34N 92°16′32E	Alpine meadow

harsh conditions (Wang, 2001; Sun and Zhu, 2000). The knowledge about patterns of natural *Kobresia* accession distribution on the Tibetan plateau and their correlation with environmental factors is very limited. In the past, the phylogenetic relationship of *Kobresia* species was studied mainly based on morphological characteristics. However, discrepancies exist among these studies. The discrepancies probably occur because morphological characters are easily influenced by environmental changes (Reznicek, 1990; Muasya et al., 1998; Starr et al., 2004).

A few molecular studies have been done on genetic relationships of Cariceae genera or species within Carex (Starr et al., 1999; Yen and Olmstead, 2000; Roalson et al., 2001), but lesser molecular studies within species of *Kobresia* was done (Zhang, 2006). Thus, molecular markers which are not sensitive to environmental conditions are needed. Molecular studies of these species will provide greater insight into their degree of genetic diversity and understanding of how they are able to thrive in a harsh environment.

Random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) are frequently used to effectively assess genetic diversity within and among accessions and to determine accession structure, even without any prior knowledge of the genome of the species (Vos et al., 1995; Hansen et al., 1999; Luo et al., 2007). In a previous study, population diversity of 11 *Kobresia* was estimated by using RAPD, yielding limited information (Zheng et al., 2009). We believe that additional studies are needed, such as comparisons of DNA sequences among these accessions, in order to better understand the phylogentic relationship among these accessions. RAPD analysis can be combined with the comparative study of nucleotide sequences to get a better resolution of the phylogenetic relationships of the *Kobresia* species (Blattner et al., 2001; Gehrig et al., 2001). NrITS, cptrnT-L-F spacer and cpndhF have proven to be informative markers for revealing phylogenetic relationships at species levels through maximum parsimony-based analyses (Olmstead and Palmer, 1994; Cerbah et al., 1998; Wen, 2000; Gehrig et al., 2001).

The main objective of the present study was to clarify the phylogenetic relationships of 11 *Kobresia* accessions collected from the Tibetan plateau using RAPD markers and the comparative analysis of the chloroplast DNA trnT-LF spacer, chloroplast DNA ndhF gene and nrDNA ITS gene sequences.

MATERIALS AND METHODS

Plant materials and DNA extraction

Eleven plant accessions representing seven different species were sampled from Tibet alpine during September, 2006. Details on these genotypes including geographic origin and geographic information system (GIS) data are provided in Table 1. Sampling vouchers were deposited at the Northwest Agriculture and Forest University Herbarium.

Genomic DNA of each accession was extracted from a pool of 20 plants using the modified cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). DNA concentration was measured using a UV spectrophotometer, and concentrations were adjusted to 50ng/µl for polymerase chain reaction (PCR) analysis.

PCR reaction and sequencing

These DNA samples were used for RAPD analysis and sequence amplification using PCR. RAPD PCR program used was: 1 cycle at 94°C for 3 min; 40 cycles at 94°C for 1 min, 37°C for 30 s, 72°C for 1 min, and was followed by a final extension at 72°C for 7 min.

The entire ITS was PCR amplified using ITS-5a (Stanford et al., 2000) and ITS-4 (White et al., 1990) primers. The whole chloroplast ndhF gene was amplified with PCR primers ndhF-F1318 and ndhF-R2110, ndhF-F1 and ndhF-R972, ndhF-F803 and ndhF-R1603 (Olmstead and Sweere, 1994), and the same ndhF primers used for PCR were used for sequencing.

The chloroplast trnL intron was PCR amplified using the trnL c and d primers (Taberlet et al., 1991). Likewise, the chloroplast trnL-trnF intergenic spacer and chloroplast trnT-trnL intergenic spacer region were amplified using the trnL e and trnF f, trnT a and trnL b primers, respectively.

Table 2. Primers and their polymorphic loci in analyzing genetic variations of 11 accessions of *Kobresia*.

Primers	Sequences 5'-3'	No. of polymorphic bands	Polymorphism (%)
S01	CCACCACGAC	16	72.3
S02	AGACGGCTCC	19	86.36
S03	ACCCGACCTG	13	59.09
S04	GTTTCGCTCC	10	45.45
S05	TGCTCTGCCC	15	68.18
S06	AGGGAACGAC	22	100
S07	GTAAACCGCC	19	86.36
S08	AGTCCGCCTG	19	86.36
S09	GAGAGGCTCC	21	95.45
S10	GGCGTATGGT	16	72.73
Average		17	67.33

PCR products were purified using the Quickstep 296-well PCR purification kit (Edge Biosystems, Gaithersburg, MD). According to the protocol (Applied Biosystems, Foster City, CA), bidirectional sequencing were performed in a 10 µl reaction volume: BigDye terminator v3.1 cycle sequencing RR-100 reagent 0.5 µl, BigDye terminator v3.1 5X sequencing buffer 2 µl, 2 µM/l primer 1 µl and purified PCR product 0.5µl. The same primers were used for PCR amplification. Products from the sequencing reactions were purified using the Performa DTR V3 96-Well short plate kit (Edge Biosystems, Gaithersburg, MD). Aqueous elutes were fractionated on an ABI3730 (Applied Biosystems, Forest City, CA) capillary electrophoresis instrument by the Center for Integrated BioSystems at Utah State University.

All sequencing PCR amplifications were performed under the following conditions: 1 cycle at 94°C for 90 s; 5 cycles at 94°C for 30 s, 55°C for 30 s (temperature decreased by 1°C for each cycle), 72°C for 1 min; 30 cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 1 min; this was followed by a final extension at 72°C for 7 min.

Data analysis

RAPD polymorphic bands were scored by quantity one software; each allele was scored as present (1) or absent (0) for each loci. The percentage of polymorphic loci, Nei's gene diversity index (Nei and Li, 1979) and Shannon's information index (Lewontin, 1972) gene flow (Nm) were calculated using PopGene32 (Yeh et al., 1997). The numerical taxonomy and multivariate analysis system (NTSYSpc 2.1) was constructed with unweighted pair group method arithmetic average (UPGMA) dendrograms of RAPD, the Mantel (1967) test statistic (Z) infer correlation of AFLP and RAPD using the Mxcomp procedure of NTSYS-pc (Rohlf, 1998). Significance tests for these correlations were determined by comparing observed values to values obtained by 1000 random permutations (Smouse et al., 1986). Therefore, the upper-tail probability (p) that 1000 random Mantel test-statistic (Z) values are (by chance) less than observed values of Z, equals 0.002 or greater.

Sequence and phylogenetic analysis

Sequencher version 4.1.4 (Genes Code, Ann Arbor, MI.), was used to assemble, inspect and edit the forward and reverse sequences. The sequences of the two regions examined were combined and aligned by Clustal W (Thompson et al., 1994), with final manual adjustment. Parsimony analysis was performed using PAUP*4.0b10 (Swofford, 1998). Ambiguous base pairs were treated as missing by default and were not used in the analysis. Heuristic parsimony searches involving 100 replications were conducted using simple sequence addition, tree bisection and reconnection (TBR) branch swapping, MaxTrees un-limited and Multrees option in effect. Branch support was evaluated as bootstrap percentages (BP) from 100 bootstrap replicates (Felsenstein, 1985) in PAUP*. A partition homogeneity test (Farris et al., 1995) with 1,000 replicates was conducted with PAUP.

RESULTS

RAPD

Ten primers were identified out of the 110 primers (Table 2). RAPD markers, which amplified 170 polymorphic bands, showed high level of polymorphism by the polymorphism information content (PIC) value ranging from 45.45 (primer S04) to 95.45% (primer 09) with an average of 67.33% across the germplasm assayed.

A matrix of the Jaccard's coefficients of similarity based on the data of RAPD markers was calculated between accessions as shown in Table 3. Pairwise comparisons among different accessions yielded values ranging from 0.509 (between accessions *Kobresia macrentha* (L) and *Kobresia humilis* (D)) to 0.832 (between accessions *Kobresia littled* (L) and *Kobresia royleana* (N)). The overall mean similarity coefficient for all possible pairwise comparisons was 0.451.

The eleven accessions can be classified into four major genetic groups when genetic similarity is 0.66 (Figure 1). *K. littled* (L), *K. littled* (N), *K. littled* (D), *K. royleana* (N), *Kobresia prattii* (D) and *K. royleana* (L) cluster together, and *K. cailli* (D), *K. humilis* (N) and *K. humilis* (D) are in one group, and accessions *K. macretha* (L) and *Kobresia pymaea* (N) do not group with any of the others.

The mean Nei's gene diversity index (H) of all accessions was 0.2221, the Shannon's information index (I) was 0.3592, indicating that genetic diversity of *Kobresia* is very rich. The gene differentiation (G_{ST}) values (0.2056) showed

Table 3. Matrix of Jaccard's coefficients of similarity between 11 accessions of *Kobresia*.

Species	*K- royleana* (L)	*K. macren* (L)	*K. littledal* (L)	*K. oylean* (N)	*K. pratti* (D)	*K. caillifol* (D)	*K. littledal* (D)	*K. littledal* (N)	*K. humilis* (N)	*K. humilis* (D)	*K. pygma* (N)
K. royleana (L)	1.000										
K. macren (L)	0.630	1.000									
K. littledal (L)	0.688	0.734	1.000								
K. roylean (N)	0.717	0.694	0.832	1.000							
K. prattii (D)	0.630	0.642	0.780	0.751	1.000						
K. caillifoli (D)	0.556	0.624	0.659	0.653	0.624	1.000					
K. littledal (D)	0.665	0.642	0.734	0.728	0.723	0.624	1.000				
K. littledal (N)	0.671	0.624	0.740	0.746	0.740	0.642	0.821	1.000			
K. humilis (N)	0.624	0.601	0.624	0.653	0.613	0.734	0.647	0.688	1.000		
K. humilis (D)	0.624	0.509	0.590	0.607	0.578	0.688	0.601	0.653	0.780	1.000	
K. pygma (N)	0.659	0.555	0.636	0.607	0.590	0.595	0.590	0.642	0.595	0.642	1.000

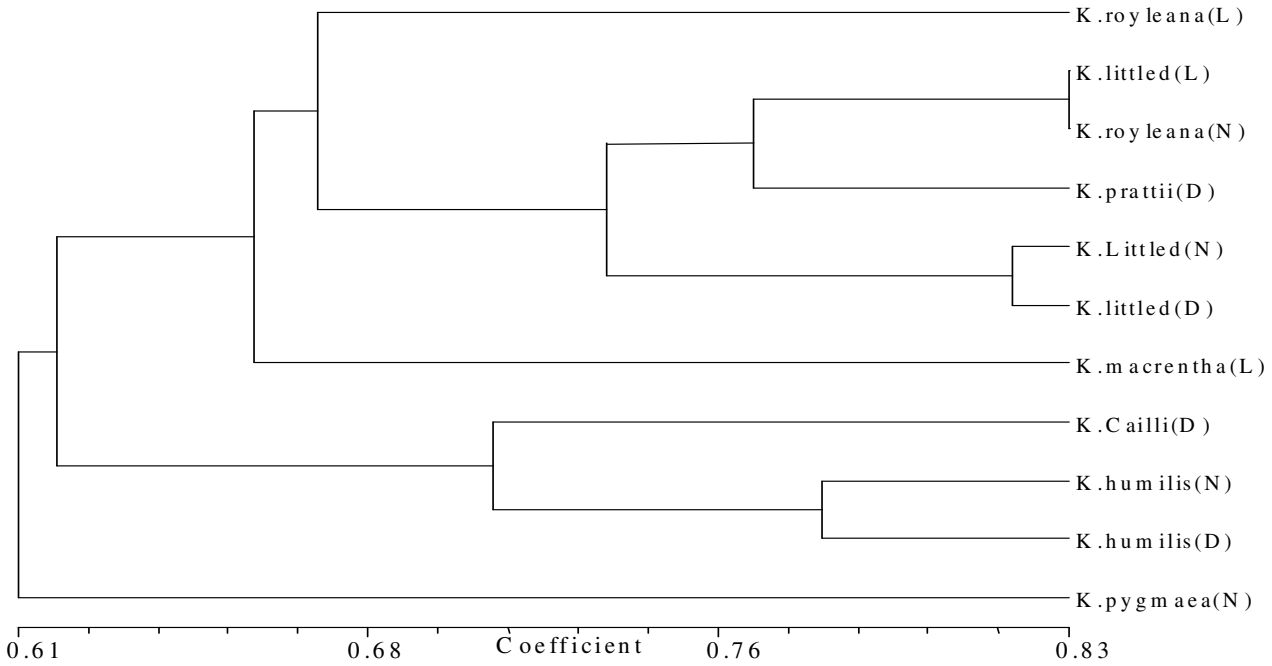

Figure 1. Genetic similarity among *Kobresia* accessions revealed by UPGMA cluster analysis based on RAPD Data.

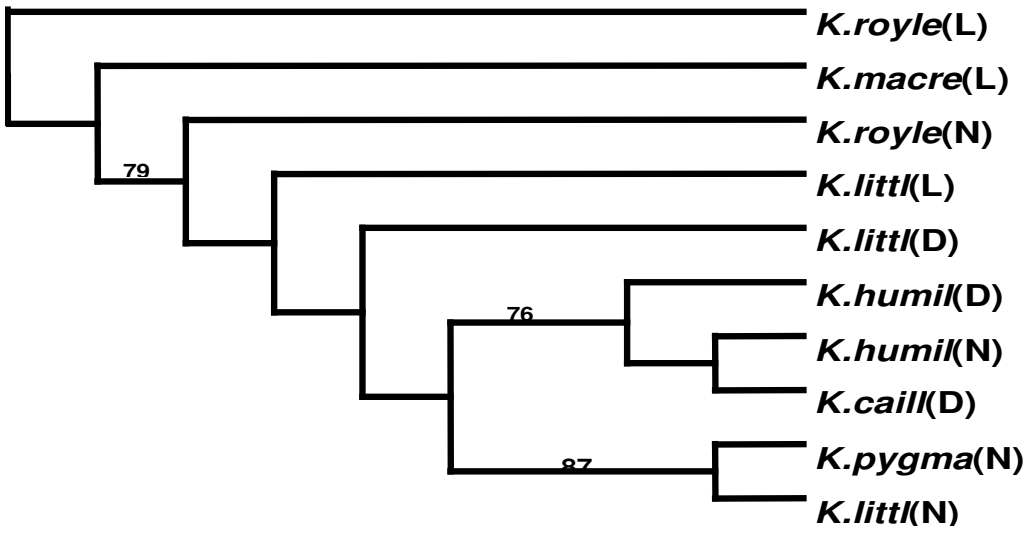

Figure 2. The most parsimonious tree based on nrITS sequence.

Table 4. Sequence characteristics of cpDNA and ITS sequences.

Sequence characteristic	ITS	cp*ndh*F	cp*trn*
Length (nucleotides) range (bp)	426 - 730	1223 - 2067	1366 - 2237
Aligned length	764	2550	3057
Parsimony informative site	97	37	107
Parsimony uninformative site	219	71	979
Tree length	462	123	1196
Consistency Index	0.8896	0.9187	0.9741
Retention index	0.6890	0.8113	0.7817

that most of the genetic variability resided among individuals within accessions, whereas, only 20.56% resided among accessions. The expected heterozygosity was calculated as total genetic diversity (Ht) and genetic diversity within all analyzed accessions (Hs). The whole material of Ht was 0.3068, Hs was 0.2437, while the gene flow (Nm) was found to be 1.9316.

Sequence results

The internal transcribed spacers (ITS) tree was divided into three clades with some internal nodes that lack bootstraps. The peculiar species *K. macrentha* (L) and *K. royleana* (N) separately form one clade, and another clade is formed by the rest of the species (Figure 2).

The characteristics of cpndhF genes are shown in Table 4. The parsimonious tree suggests that there are three primary clades: *Kobresia caillifolia* (D) and *K. humilis* (D) composing one clade, another clade containing only *K. macrentha* (L), while the rest of the species form the third clade (69% BS) (Figure 3).

The sequence characteristics for the region cptrnT-L-F are described in Table 4. The trnT-trnF region sequences form three clades (Figure 4): *Kobresia pygmaea* (D) and *K. macrentha* (L) formed one clade with a weak BP value; *Kobresia littledalei* (N), *K. littledalei* (D) and *K. prattii* (D) cluster together with a moderately supporting value (86% BS); the rest of the species form a very weakly supported group (59% BS). Within the clade, very good BP values support subgroup *K. royleana* (L) and *K. royleana* (N) (99% BS), *K. humilis* (N) and *K. humilis* (D) (96% BS).

Analysis using combined sequences

In the partition homogeneity test, there was no significant incongruence among cpDNA sequences ($P > 0.05$). To obtain more information from the sequences, the two data sets were combined and analyzed. Heuristic parsimony searches for the combined cpndhF and cptrnT-trnF data sets yielded one tree, of which 144 were parsimony informative (length = 1328; CI = 0.96; RI=0.74).

The strict consensus tree (Figure 5) showed 3 main

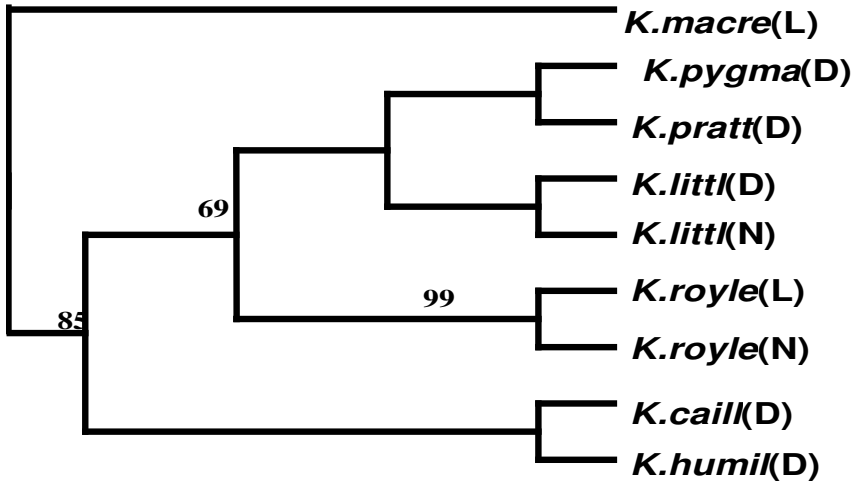

Figure 3. The most parsimonious tree based on cpndhF sequence

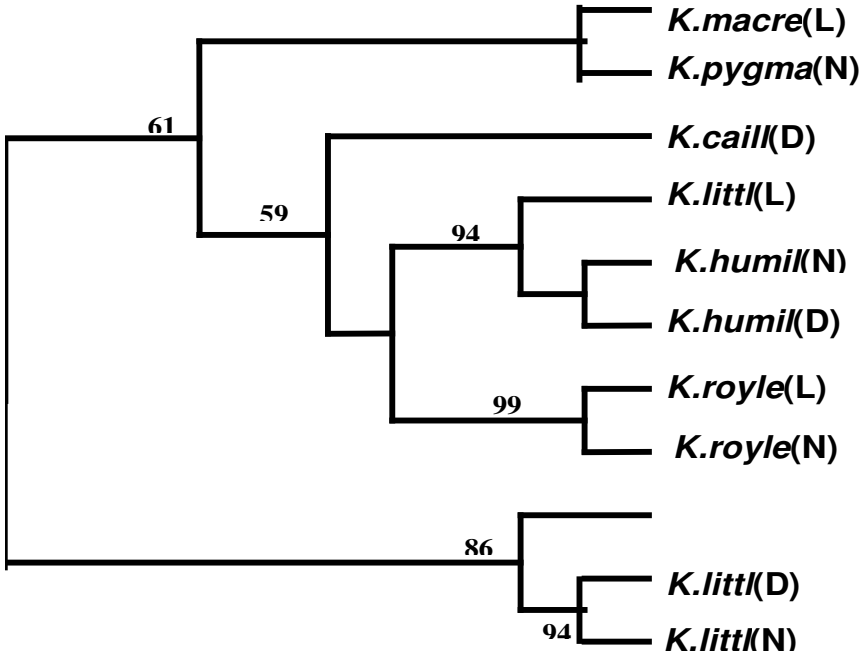

Figure 4. The most parsimonious tree based on cptrnT-L-F sequence.

clades. The first clade (67% BS) consisted of *K. littledalei* (L), *K. pygmaea* (N), *K. humilis* (N), *K. humilis* (D) and *K. caillifolia* (D). Within this clade, *K. humilis* (N) and *K. humilis* (D) formed a strongly supported (100% BS) group. The second moderate clade (72% BS) was divided into two parts, a strong clade (99% BS) containing *K. royleana* (L) , *K. royleana* (N), and a moderate clade (82% BS) including *K. prattii* (D), *K. littledalei* (D) and *K. littledalei* (N). *K. macrentha* (L) formed an upper cluster by itself and is remote from other species.

DISCUSSION

Sequences

Kobresia is a wind pollinated plant which has reduced floral structures that eliminate many "key" characters that are useful in elucidating phylogenetic relationships. Thus, each accession as shown in the dendrogram may be comprised of several more or less different genotypes. Whether these differences stem from sexual recombination or from mutations still needs to be determined.

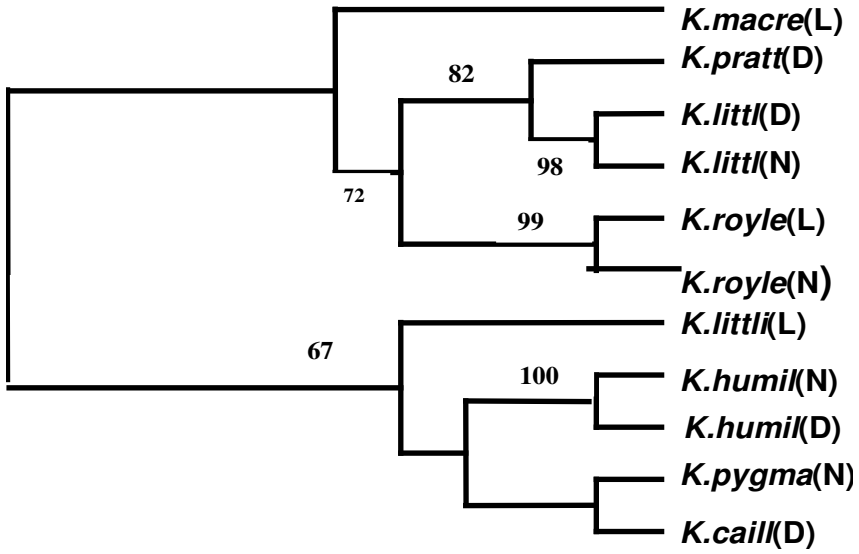

Figure 5. The most parsimonious tree based on cpndhF and cptrnT –trnF sequence.

All accessions of the same species cluster together except *K. littledale* (L) supported by cpDNA sequence. Accessions that are separated by great distances with different environment conditions may have influenced the genotype. It is also possible due to misidentification, since GenBank blast showed that *K. littledale* (L) is similar to *Festuca* based on cpndhF and cptrn intron sequences (Table 5). *Festuca* is a grass that is different from *Carex* or *Kobresia*. Generic delimitation within *Kobresia* is based largely on the morphology of the inflorescence structure. Blurring of the generic boundaries becomes a problem for some taxa having morphological characters that are interpreted as intermediate.

The structure of the combined analysis strict consensus tree (Figure 5) is most similar to the cpndhF strict consensus tree in terms of internal branch structure. CpDNA dendrograms are mostly congruent with the ITS tree. The primary difference between the results is that the accessions *K. royleana* (L) and *K. royleana* (N) cluster together in the cptrnT-L-F and cpndhF gene analysis, whereas, the nrITS analysis places two accessions separately. Nuclear DNA is biparentally inherited and chloroplastic DNA is maternally inherited, so the difference most likely results from a hybridization or allopolyploidization process as more than two species often coexist in the same ecological niche. Growing evidence shows that ITS polymorphism or incomplete homogenization is useful for understanding the origin of hybrids and polyploidy species (Bailey et al., 2003, Liu et al., 2006).

RAPD

Our study revealed that the genetic diversity of *Kobresia* accession levels from RAPD (H = 0.2221 and I = 0.3592) was relatively lower than the levels reported using AFLP (H = 0.2430 and I = 0.4012) in a previous study (Zheng et al., 2009). The Mantel test showed a significant correlation between the RAPD and AFLP-based genetic similarity (r = 0.65528, p=0.002), and was found to be very useful for genetic diversity study in *Kobresia*.

A percentage of polymorphic bands (PPB) around 50% is usually regarded as high genetic diversity (Ma et al., 2000). The resulting polymorphic locus obtained from this study revealed by RAPD (67.33%) is lower than that revealed by AFLP (93.96%) for *Kobresia* accessions. Both AFLP and RAPD showed a relatively high level of genetic diversity. *Kobresia* species are perennial herbs. In order to adapt to low temperatures, short growing seasons and low rainfall on the Tibetan plateau (Zhou and Deng, 2001), the recruitment of sexual progeny is extremely rare and propagation occurs predominantly through clonal growth. Studies suggest that even low rates of seedling recruitment are sufficient in maintaining high levels of genetic diversity (Soane and Watkinson, 1979; Watkinson and Powell, 1993; Zhou and Deng, 2001). Sexual reproduction may be a possible explanation for the relatively high genetic diversity in these clonal plants (Torimaru et al., 2003).

The *Kobresia* mean Nei's gene diversity index (H) was 0.2221, and the Shannon's information index (I) was 0.3592. This indicates that the genetic diversity of *Kobresia* is very rich. Nybom and Bartish (2000) reported an average RAPD-based G_{ST} value of 0.23 for some long-lived perennials and seed wind species. The value (0.2056) is lower than average, and shows that only 20.56% resided among accessions.

Assessment of genetic variability and its partitioning is

Table 5. The results of GenBank blast.

Species	ITS (Max Identity %)	*ndh*F (Max Identity %)
K. royleana (L)	*Unicinia multifaria* (100)	*Kobresia nepalensis* (98)
K. macren (L)	*Fimbristylis microcarya* (100)	*Scirpus microcarpus* (98)
K. littledal (L)	*Schoenoxiphium filiforme* (96)	*Festuca subverticillate* (96)
K. roylean (N)	*Carex nigricans* (97)	*Kobresia nepalensis* (98)
K. prattii (D)	*Uncinia phleoides* (100)	*Kobresia nepalensis* (99)
K. caillifoli (D)	*Carex maritime* (97)	*Kobresia nepalensis* (98)
K. littledal (D)	*Schoenoxiphum lanceum* (97)	*Kobresia nepalensis* (99)
K. littledal (N)	*Kobresia sibirica* (98)	*Kobresia nepalensis* (99)
K. humilis (N)	*Carex maritime* (100)	------
K. humilis (D)	*Carex incurviformis* (97)	*Kobresia nepalensis* (98)
K. pygma (N)	*Kobresia sibirica* (98)	*Kobresia nepalensis* (99)
	***Trn*T-Lintergenic spacer**	***Trn*L-F intergenic spacer**
K. royleana (L)	*Kobresia myosuroides* (98)	*Carex supina* (98)
K. macren (L)	*Kobresia myosuroides* (98)	*Blysmus compressus* (88)
K. littledal (L)	*Carex bohemica* (100)	*Kobresia capillifolia* (94)
K. roylean (N)	*Kobresia myosuroides* (98)	*Kobresia simpliciuscula* (98)
K. prattii (D)	----------	*Kobresia simpliciuscula* (97)
K. caillifoli (D)	*Kobresia myosuroides* (98)	*Kobresia sibirica* (97)
K. littledal (D)	*Carex tomentosa* (100)	*Kobresia myosuroides* (99)
K. littledal (N)	*Carex aurea* (100)	*Carex elvnoides* (97)
K. humilis (N)	*Carex bohemica* (100)	*Carex ovalis* (98)
K. humilis (D)	*Carex bohemica* (100)	*Carex ovalis* (98)
K. pygma (N)	*Kobresia myosuroides* (98)	*Kobresia myosuroides* (98)

a major concern of the study involved in for example, life history traits, breeding system, successional status, or conservation genetics. This study is similar to previous studies that have demonstrated that the vagility of pollen and seeds is highly associated with the development of genetic structure (Zhou and Deng, 2001; Zhao et al., 2006).

Conclusion

The marker RAPD was found equally informative and useful for a better understanding of the genetic variability and genomic relationships between accessions. In the dendrograms, *K. macrentha* (L) species formed a particular clade, and species inhabiting similar habitats or having similar adaptations tended to be together. These trees show that the genetic variation differences and adaptations may possibly be taking place in these *Kobresia* accessions because of mutations or drift effects due to their remote geographic and high altitudinal position in the Tibetan plateau.

Finally, this study highlights the importance of molecular analysis in understanding the genetic diversity and structure of *Kobresia* accessions, and contributes to the knowledge of genetic resource conservation.

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