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Genetic diversity in populations of the medicinal plant *Leonurus cardiaca* L. revealed by inter-primer binding site (iPBS) markers

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Abstract Motherwort (*Leonurus cardiaca* L.) is a medicinal plant indigenous to the Mediterranean regions in Europe and Asia. The objective of this study is to apply inter-primer binding site (iPBS) markers to assess the molecular variation and genetic relationships of 89 genotypes of motherwort to assist the genetic improvement of this species. The genotypes comprised 79 from Iran and 10 collected in Australia and 15 additional accessions of two related species (*L. heterophyllus* Sweet and *L. sibiricus* L.) collected in Australia, were also included. PCR of 7 iPBS primers (dominant markers) produced a total of 191 bands ranging from 180 to 4000 bp and the mean PIC for primers ranged from 0.2213 to 0.3206 with a mean value 0.2921. The mean expected heterozygosity (0.134), the mean unbiased expected heterozygosity (0.140) and Shannon's information index (0.213) indicated a high level of inbreeding among the accessions tested. Ordination and cluster analysis showed that the genetic relationships among all accessions could be separated into three major

groups—*L. cardiaca*, *L. heterophyllus* and *L. sibiricus*. However, among the 89 accessions of *L. cardiaca*, genotypes collected from the same geographic region tended to cluster together thus indicating greater genetic similarity. The Motherwort accessions originating in Iran are highly divergent and possess abundant genetic diversity and clearly provide a basis for selection and breeding. The iPBS PCR-based genome fingerprinting technology used in this study is low-cost and effective in differentiating accessions of motherwort and their related species.

Keywords Genetic diversity · Germplasm · *Leonurus cardiaca* · *L. heterophyllus* · Motherwort · Plant breeding

Introduction

Motherwort (*Leonurus*) belongs to the Mint family (Lamiaceae) and most species in the genus are native to Europe and Asia within the North Temperate Zone (Wu and Li 1977). The genus *Leonurus* includes 24 species with 3 divisions and 5 sub-divisions (Ahvazi et al. 2012; Chao and Zhou 1998; Erdtman 1945; Hedge 1990; Li and Hedge 1994). There are 12 species of the genus *Leonurus* distributed across China, commonly named *yimǔcǎo*, which literally means 'beneficial herb for mothers' (Wu and Li 1977). For more than 2000 years the Chinese have used certain

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Leonurus species including *L. chaituroides* Wu et Li, *L. heterophyllus* Sweet *L. sibiricus* L. as medicinal herbs to invigorate blood circulation, regulate menstrual function, dissolve blood stasis, generate new tissues, promote urine excretion and reduce inflammation (Chinese Pharmacopoeia Commission 2010; Yin et al. 2010).

Recent pharmaceutical research revealed that the chemical constituents of *Leonurus cardiaca* L., *Leonurus heterophyllus* Sweet and *Leonurus sibiricus* L. include flavonoids (apigenin, genkwanin), triterpenoids (ursolic acid and corosolic acid), alkaloids (stachydrine, leonurine), isoquerestrine and betonicine (Tomasbarberan et al. 1993; Ulubelen et al. 2005; Janicsak et al. 2006; Ali et al. 2007). Motherwort is also rich in epi-cedrol, a-humulene, germacrene-D and spathulenol for medicinal use (Morteza-Semnani et al. 2008). Nowadays, products made from motherwort have been used extensively to treat psychological disorders, heart ailments, premenstrual syndrome (PMS), high blood pressure, stresses and labor pain. These benefits are attributed to the extracted chemicals that strengthen the nervous system, reduce spasms, increase blood circulation and reduce thyroid hormone production (Arber 1938; Chao and Zhou 1998; EMA/HMPC 2010; Mills and Bone 2000; Popescu et al. 2009; Quattrocchi 2012; WHO 1998).

Common Motherwort (*L. cardiaca*) is an herbaceous perennial plant. The morphological characteristics of this species were described by Wu and Li (1977) and Hedge (1990). The species originated in northern Europe and subsequently spread to Western Siberia and other parts of northern Asia (Omidbagi 2010; Paine and Ribic 2002), evolving extensively in Iran (Ahvazi et al. 2012; Mozaffarian 1996). Early morphological studies indicated that motherwort populations collected from different regions in Iran were diverse phenotypically (Mozaffarian 1996; Milkowska-Leyck et al. 2002). The Siberian motherwort (*L. sibiricus*) or honey-weed is an herbaceous annual or biennial herb native to the Siberia, Mongolia and Northern China. The major morphological characteristics of this species include squared stems in cross sections and 3-angled glabrous fruits (Wu and Li 1977). The Chinese motherwort (*Leonurus heterophyllus*) also called *L. japonicus*, is an herbaceous plant native to China, Korea, Japan, Cambodia and northern Australia (Chao and Zhou 1998; Quattrocchi 2012; Wu and Li 1977). This species is an erect sub-

shrub with many lateral branches and it blooms from June to September in China (Wu and Li 1977).

Studies on population genetic diversity on motherworts are very limited. An AFLP marker system was initially used to study the genetic diversity of nine accessions of *L. japonicus* germplasm (Khadivi-Khub and Soorni 2014; Yu et al. 2009). Chen et al. (2009) later used an ISSR marker system on 19 accessions of *L. japonica* collected from 19 different provinces in China. They concluded that three distinct genetic groups existed. Ren (2012) designed SSR primers and 10 pairs of SCoT primers and used these to differentiate 66 accessions of *L. heterophyllus*. Recently, Khadivi-Khub and Soorni (2014) adopted a combination of AFLP, ISSR, RAPD, IRAP marker systems to analyze *L. cardiaca* accessions collected from a few areas in Iran. Result showed that accessions collected from a particular region were clustered as an individual group, and accessions collected from the Dargaz region were genetically distant to all the other populations.

iPBS markers were recently developed as an alternative method to explore genetic diversity and relationships in plants (Alzohairy et al. 2014; Kalendar et al. 2010, 2011; Smykal et al. 2011). As a dominant marker system, the iPBS requires no previous knowledge of the genome (Alzohairy et al. 2014; Odong et al. 2011). Due to the limited genomic information available for genus *Leonurus*, we adopted the iPBS marker system in this study. This marker system was used to fingerprint apricot (*Prunus armeniaca*) (Baranek et al. 2012), guava (*Psidium guajava* L.) (Mehmood et al. 2013; Mehmood et al. 2015), grapes (*Vitis vinifera* L.) (Guo et al. 2014) and cordyline (*Cordyline hybrida*) (Luo et al. 2015). The objectives of this study are to investigate the genetic diversity and relationships of Iranian and Australian motherwort germplasm (89 *L. cardiaca*, 10 *L. heterophyllus* and 5 *L. sibiricus*) using the new iPBS technique to provide a basis for targeted genetic improvement of these species in both countries.

Materials and methods

Plant materials

The plant materials used in this study and their origins are listed in Table 1. One hundred and four accessions,

including 10 accessions of *L. heterophyllus* and five of *L. sibiricus*, were used for iPBS analysis. Seeds of 79 accessions of *L. cardiaca* were collected from five different areas of Iran including the provinces of Kerman (14), Taleghan (8), Sarab (24), Khansar (23) and Dargaz (10). Seeds of another 25 accessions including *L. cardiaca* (10), *L. heterophyllus* (10) and *L. sibiricus* (5) were bought from Royston Petrie, Beautanicals and All Rare Herbs, respectively. Plants were grown for tissue sampling at The Plant Breeding Institute—Cobbitty, University of Sydney in 2015.

DNA extraction and quantification

Fresh young leaves were selected for DNA isolation. The DNA was extracted from 200 mg of fresh leaves using the plant DNA isolation Mini Kit (Bioline, Australia) in accordance with the manufacturer's protocols. Quality and quantity of DNA was checked following 2.0 % agarose gel electrophoresis by comparison with known λ DNA concentrations. A portion of the isolated DNA was diluted in molecular grade water to 10 ng/ μ L concentration and used as a template for PCR.

iPBS PCR amplification

The iPBS primers listed by Kalendar et al. (2010) and used in this study were obtained from Sigma Aldrich (Castle Hill, NSW, Australia). DNA amplification was conducted using a slightly modified protocol from Kalendar et al. (2010). PCR was performed in 20 μ L reaction mixtures containing 10 ng genomic DNA, 1 time GoTaq buffer (Promega), 0.5 μ M of primer (single primer), 0.2 mM dNTPs, 0.5 U Taq DNA polymerase (GoTaq, Promega) and 2.0 mM MgCl₂. The PCR program had an initial hot start at 95 °C for 3 min, 42 cycles of denaturation at 94 °C for 30 s, annealing at 31–58 °C for 30 s and with an extension at 72 °C for 2 min. There was a final extension at 72 °C for 5 min and the program was terminated by holding at 10 °C. The reaction was performed in a Bio-Rad T100™ Thermal Cycler with 0.2 mL tubes or 96-well plates. A 10 μ L sample of each PCR product was electrophoresed at 70 V for 3.5 h in a 1.5 % (w/v) thin agarose gel with 1 \times TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). A Thermo Scientific GeneRuler 1 kb ladder (Fermentas, Australia) was used to estimate fragment lengths. Gels

were post-stained with GelRed (Biotium) for 15–20 min and photographed using the BIO-RAD Gel Doc™ XR+ with Imaging Lab™ Software.

Data scoring and analysis

PCR was performed three times for each primer to confirm band pattern consistency. DNA bands were sized and scored using Imaging Lab™ Software and carefully checked manually. Only clear bands were scored and faint bands were ignored. Bands with the same size were assumed to represent a single locus. Data were recorded for each locus using '1' for presence of a band and '0' for absence to build a binary matrix.

Summary statistics for each group of accessions relating to allelic richness, heterozygosity, genetic diversity, number of alleles and Shannon's Information Index were computed using GenAIEx 6.5 (Peakall and Smouse 2006, 2012). Shannon's Information Index was calculated following the method of Lewontin (1972). Duplicates in the data set were checked by multi-locus matching. Accessions with different names that were completely matched at all the loci were considered as duplicates. Pair-wise genetic distance was calculated using the DISTANCE procedure contained in GenAIEx 6.5. This program was also used for principal coordinate analysis (PCoA). Dendrograms were constructed based on a Dice genetic similarity coefficient (Nei and Li 1979) using the un-weighted pair-group method with arithmetic averages (UPGMA). The matrix data were imported into Tree Drawing software from PHYLIP (Felsenstein 2005) for dendrogram construction (Fig. 1).

Results

DNA polymorphism for 7 iPBS primers

Sixty iPBS primers were initially screened for polymorphism using one DNA accession and 35 primers generated a PCR product with a varied number of bands. These 35 primers were further screened for polymorphism using four DNA samples (A1, Kh1, H1 and Si1) and seven primers (2076, 2083, 2380, 2382, 2389, 2390 and 2391) were subsequently selected for iPBS PCR amplifications based on the large number of polymorphic bands they generated (Table 2). The sizes of reproducible and scorable bands ranged from

Table 1 One hundred and four motherwort (*Leonurus cardiaca* L.) accessions used in iPBS analysis

Accessions	Origin	Species name	Characters
K1	Madoon, Kerman, Iran	<i>L. cardiaca</i> L.	Delicate leaves
K2	Madoon, Kerman, Iran	<i>L. cardiaca</i> L.	Delicate leaves
K3	Madoon, Kerman, Iran	<i>L. cardiaca</i> L.	Delicate leaves
K4	Madoon, Kerman, Iran	<i>L. cardiaca</i> L.	Delicate leaves
K5	Madoon, Kerman, Iran	<i>L. cardiaca</i> L.	Delicate leaves
K6	Madoon, Kerman, Iran	<i>L. cardiaca</i> L.	Delicate leaves
K7	Madoon, Kerman, Iran	<i>L. cardiaca</i> L.	Delicate leaves
K8	Madoon, Kerman, Iran	<i>L. cardiaca</i> L.	Delicate leaves
K9	Madoon, Kerman, Iran	<i>L. cardiaca</i> L.	Delicate leaves
K10	Madoon, Kerman, Iran	<i>L. cardiaca</i> L.	Delicate leaves
K11	Madoon, Kerman, Iran	<i>L. cardiaca</i> L.	Delicate leaves
K12	Madoon, Kerman, Iran	<i>L. cardiaca</i> L.	Delicate leaves
K13	Madoon, Kerman, Iran	<i>L. cardiaca</i> L.	Delicate leaves
K14	Madoon, Kerman, Iran	<i>L. cardiaca</i> L.	Delicate leaves
T1	Taleghan, Alborz, Iran	<i>L. cardiaca</i> L.	Deep loopes
T2	Taleghan, Alborz, Iran	<i>L. cardiaca</i> L.	Deep loopes
T3	Taleghan, Alborz, Iran	<i>L. cardiaca</i> L.	Deep loopes
T4	Taleghan, Alborz, Iran	<i>L. cardiaca</i> L.	Deep loopes
T5	Taleghan, Alborz, Iran	<i>L. cardiaca</i> L.	Deep loopes
T6	Taleghan, Alborz, Iran	<i>L. cardiaca</i> L.	Deep loopes
T7	Taleghan, Alborz, Iran	<i>L. cardiaca</i> L.	Deep loopes
T8	Taleghan, Alborz, Iran	<i>L. cardiaca</i> L.	Deep loopes
S1	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S2	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S3	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S4	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S5	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S6	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S7	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S8	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S9	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S10	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S11	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S12	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S13	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S14	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S15	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S16	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S17	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S18	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S19	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S20	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S21	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S22	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S23	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves
S24	Sarab, Aldebil, Iran	<i>L. cardiaca</i> L.	Thick leaves

Table 1 continued

Accessions	Origin	Species name	Characters
Kh1	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh2	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh3	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh4	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh5	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh6	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh7	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh8	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh9	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh10	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh11	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh12	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh13	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh14	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh15	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh16	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh17	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh18	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh19	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh20	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh21	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh22	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
Kh23	Khansar, Isfahan, Iran	<i>L. cardiaca</i> L.	Round leaves
D1	Dargaz, South-Khorasan, Iran	<i>L. cardiaca</i> L.	Dwarf plant
D2	Dargaz, South-Khorasan, Iran	<i>L. cardiaca</i> L.	Dwarf plant
D3	Dargaz, South-Khorasan, Iran	<i>L. cardiaca</i> L.	Dwarf plant
D4	Dargaz, South-Khorasan, Iran	<i>L. cardiaca</i> L.	Dwarf plant
D5	Dargaz, South-Khorasan, Iran	<i>L. cardiaca</i> L.	Dwarf plant
D6	Dargaz, South-Khorasan, Iran	<i>L. cardiaca</i> L.	Dwarf plant
D7	Dargaz, South-Khorasan, Iran	<i>L. cardiaca</i> L.	Dwarf plant
D8	Dargaz, South-Khorasan, Iran	<i>L. cardiaca</i> L.	Dwarf plant
D9	Dargaz, South-Khorasan, Iran	<i>L. cardiaca</i> L.	Dwarf plant
D10	Dargaz, South-Khorasan, Iran	<i>L. cardiaca</i> L.	Dwarf plant
A1	Australia	<i>L. cardiaca</i> L.	Shiny leaves
A2	Australia	<i>L. cardiaca</i> L.	Shiny leaves
A3	Australia	<i>L. cardiaca</i> L.	Shiny leaves
A4	Australia	<i>L. cardiaca</i> L.	Shiny leaves
A5	Australia	<i>L. cardiaca</i> L.	Shiny leaves
A6	Australia	<i>L. cardiaca</i> L.	Shiny leaves
A7	Australia	<i>L. cardiaca</i> L.	Shiny leaves
A8	Australia	<i>L. cardiaca</i> L.	Shiny leaves
A9	Australia	<i>L. cardiaca</i> L.	Shiny leaves
A10	Australia	<i>L. cardiaca</i> L.	Shiny leaves
H1	Australia	<i>L. heterophyllus</i> Sweet	Big leaves
H2	Australia	<i>L. heterophyllus</i> Sweet	Big leaves
H3	Australia	<i>L. heterophyllus</i> Sweet	Big leaves

Table 1 continued

Accessions	Origin	Species name	Characters
H4	Australia	<i>L. heterophyllus</i> Sweet	Big leaves
H5	Australia	<i>L. heterophyllus</i> Sweet	Big leaves
H6	Australia	<i>L. heterophyllus</i> Sweet	Big leaves
H7	Australia	<i>L. heterophyllus</i> Sweet	Big leaves
H8	Australia	<i>L. heterophyllus</i> Sweet	Big leaves
H9	Australia	<i>L. heterophyllus</i> Sweet	Big leaves
H10	Australia	<i>L. heterophyllus</i> Sweet	Big leaves
Si1	Australia	<i>L. sibiricus</i> L.	Big leaves
Si2	Australia	<i>L. sibiricus</i> L.	Big leaves
Si3	Australia	<i>L. sibiricus</i> L.	Big leaves
Si4	Australia	<i>L. sibiricus</i> L.	Big leaves
Si5	Australia	<i>L. sibiricus</i> L.	Big leaves



Fig. 1 Geographic location of collection sites of *L. cardiaca* populations in Iran. The geographic sites including location and province in Iran are listed as numbers

180 to 4000 bp. The iPBS fingerprinting pattern of the 30 accessions from primer 2380 are shown in Fig. 2. The number of unique banding patterns among these 104 accessions validated the use of iPBS markers for the identification of motherwort DNA.

The seven primers used amplified a total of 191 scorable bands indicating a high degree of genetic variability. The information from these seven primers, including band polymorphism and mean PIC (polymorphic information content) values, is included in Table 2. Primer 2390 produced the highest number of bands (32) and primer 2391 generated the lowest (20). Primer 2380 had the highest PIC value (0.3206) whereas primer 2391 had the lowest (0.2213). The mean PIC value for these seven primers was 0.2921. These results indicate that the iPBS marker system can reveal a wide range of genomic DNA diversity in motherwort (*L. cardiaca*) and its related species.

Primer 2380 generated a specific band (~550 bp) for five accessions (100–104) of *L. sibiricus* with large leaves (Fig. 2; Table 1). Primer 2390 produced a common band (~1100 bp) for both *L. heterophyllus* and *L. sibiricus* accessions (90–104) with large leaves. Primer 2390 also amplified a common band (~1350 bp) for the population of 10 accessions (80–89) of *L. cardiaca* with shiny leaves collected from the seed company Royston Petrie (Fig. 3; Table 1). Primer 2391 generated a specific band (~1300 bp) for the population of 10 accessions (70–79) of *L. cardiaca* collected from Dargaz in Iran. All 10 accessions of *L. cardiaca* originating from Dargaz are dwarf in stature. Primer 2391 amplified another three specific bands (~300 bp, ~500 bp and

~800 bp) for both *L. heterophyllus* and *L. sibiricus* accessions (90–104) with large leaves.

Heterozygosity and diversity of species

Summary statistics for each of the 8 groups of accessions [K (Kerman), T (Teleghan), S (Sarab), Kh (Khansar), D (Dargaz), A (Australia), H (*L. heterophyllus*) and Si (*L. sibiricus*)] including number of different alleles, number of effective alleles, Shannon's information index, expected heterozygosity and unbiased expected heterozygosity are listed in Table 3. Expected heterozygosity values (H_e) ranged from 0.083 (A) to 0.172 (S), with an average of 0.134, whereas unbiased expected heterozygosity (uH_e) ranged from 0.088 (A) to 0.175 (S) with an average of 0.140. The Shannon's information index among the 8 groups ranged from 0.131(A) to 0.286 (S), with an average of 0.213. The percentage of polymorphic loci (PPL) for *L. sibiricus* and *L. heterophyllus* were the lowest at 27.80 % and 30.94 %, respectively. Among the six populations of *L. cardiaca*, the PPL value ranged from 31.39 (A) to 84.30 % (S), with an average of 60.16 %. The PPL results among five populations from Iran varied from 48.88 (D) to 84.30 % (S) with a mean value of 65.92 %.

The Nei genetic distances for eight populations of motherwort are listed in Table 4. Among all five populations of *L. cardiaca* from Iran, the smallest genetic distances existed between S/Kh (0.021) and K/T (0.022), while the greatest genetic distances were observed between K/D (0.100) and T/D (0.103). The genetic distance between the Australian (A) population and five of the Iranian populations varied from A/S

Table 2 Seven iPBS primers used in the detection of polymorphism among 104 accessions of *Leonurus* spp.

iPBS primer	Sequence (5'–3')	Ta (°C)	Number of bands ^a	Mean PIC value ^b
2076	GCTCCGATGCCA	50	31 (31)	0.3119 (0.2652)
2389	ACATCCTTCCCA	38	30 (30)	0.3062 (0.2770)
2380	CAACCTGATCCA	38	30 (30)	0.3028 (0.2772)
2390	GCAACAACCCCA	45	22 (20)	0.3206 (0.2700)
2391	ATCTGTGACCCA	38	20 (18)	0.3045 (0.2561)
2083	CTTCTAGCGCCA	41	26 (25)	0.2213 (0.1861)
2382	TGTTGGCTTCCA	41	32 (30)	0.2772 (0.2481)

Results in brackets are only for 89 accessions of *L. cardiaca*

^a Total accountable bands consistently appearing in two or three repeated experiments

^b PIC value is calculated as $PIC = 1 - [f^2 + (1 - f)^2]$, where f is the frequency of the marker in the data set

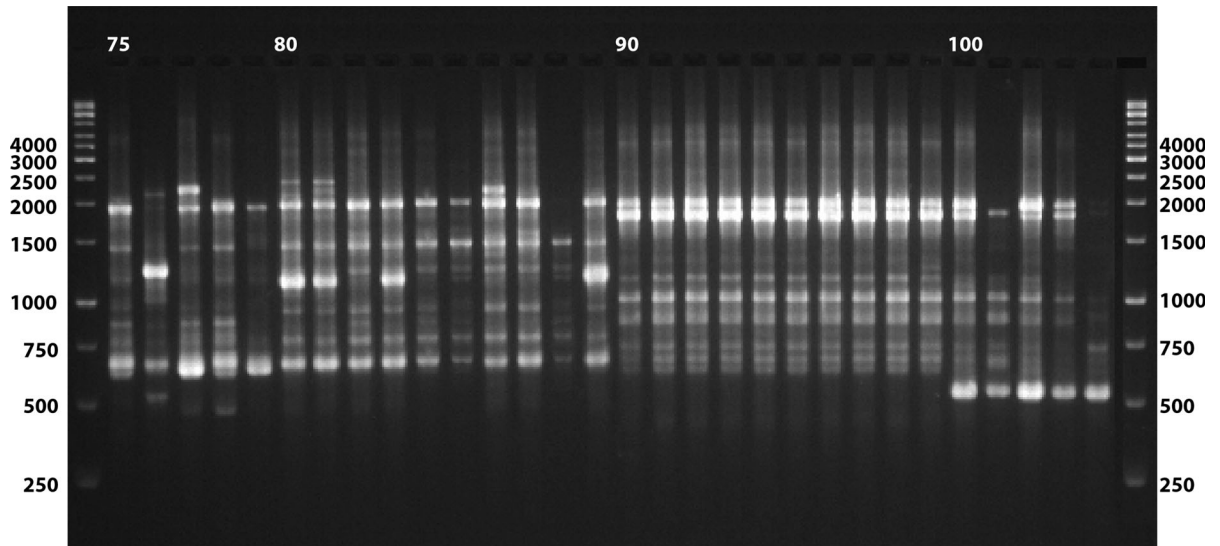


Fig. 2 PCR banding pattern for 30 Motherwort (*Leonurus cardiaca* L.) accessions using primer 2380

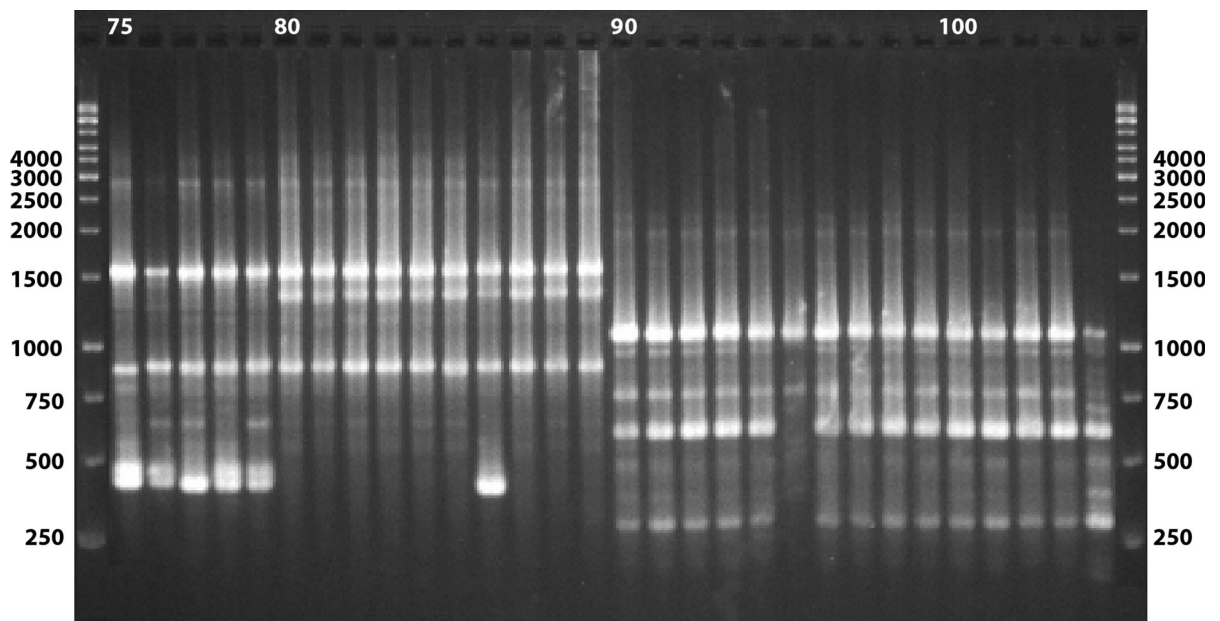


Fig. 3 Species specific banding pattern for 30 DNA accessions using iPBS primer 2390

(0.106) to A/D (0.136). These results show that the Australian (A) population of *L. cardiaca* is genetically distant from the Iranian materials. The genetic distance between the *L. sibiricus* population and the six *L. cardiaca* populations was even greater and ranged from Si/S (0.163) to Si/A (0.252). The genetic

distance between the *L. heterophyllus* population and the six *L. cardiaca* populations was large and ranged from H/D (0.203) to H/A (0.295). However, the genetic distance between *L. heterophyllus* and *L. sibiricus* was only 0.074; indicating a close genetic relationship between these two species.

Table 3 Summary statistics for 104 motherwort (*Leonurus* spp.) accessions assessed with 7 iPBS primers (Kalendar et al. 2010)

Populations	N	Na	Ne	I	He	uHe	PPL (%)
K	14.000	1.300	1.235	0.242	0.150	0.156	63.23
T	8.000	1.152	1.258	0.251	0.160	0.171	56.95
S	24.000	1.695	1.254	0.286	0.172	0.175	84.30
Kh	23.000	1.552	1.255	0.278	0.169	0.173	76.23
D	10.000	1.031	1.235	0.222	0.143	0.151	48.88
AC	10.000	0.717	1.135	0.131	0.083	0.088	31.39
H	10.000	0.749	1.162	0.148	0.097	0.102	30.94
Si	5.000	0.659	1.163	0.144	0.096	0.106	27.80
Mean	13.000	1.107	1.212	0.213	0.134	0.140	52.40

N number of sample size, *Na* number of different alleles, *Ne* number of effective alleles, *I* Shannon's information index, *He* expected heterozygosity, *uHe* unbiased expected heterozygosity, *PPL* percentage of polymorphic loci, *K* Kerman, *T* Teleghan, *S* Sarab, *Kh* Khansar, *D* Dargaz, *A* Australia, *H* *L. heterophyllus* and *Si* *L. sibiricus*

Table 4 Pairwise population matrix of Nei genetic distance for eight groups of motherworts

K Kerman, *T* Teleghan, *S* Sarab, *Kh* Khansar, *D* Dargaz, *A* Australia, *H* *L. heterophyllus* and *Si* *L. sibiricus*

	K	T	S	Kh	D	A	H	Si
K	0.000							
T	0.022	0.000						
S	0.036	0.030	0.000					
Kh	0.037	0.035	0.021	0.000				
D	0.100	0.103	0.079	0.082	0.000			
A	0.128	0.122	0.106	0.124	0.136	0.000		
H	0.224	0.216	0.213	0.225	0.203	0.295	0.000	
Si	0.176	0.165	0.163	0.178	0.172	0.252	0.074	0.000

Principal component analysis (PCA) for seven iPBS markers

Principal coordinate analysis (PCoA) demonstrated spatially the relative genetic distances among the individual accessions and revealed six distinct groups (Fig. 4). The plane of the first three PCoA axes accounted for 31.54 % of the total variation (first axis = 14.84 %, second = 9.0 %, third = 7.70 %). Ten accessions of *L. heterophyllus* and 5 accessions of *L. sibiricus* clustered separately on the lower left plane. The D population (10 accessions) was distributed on the upper left plane and the A population (10 accessions) was scattered on the upper right plane. The T population (8 accessions) and the K population (14 accessions) clustered together at the lower right plane. Both the S population (24 accessions) and the Kh population (23 accessions) were distributed across the whole right plane.

Dendrogram generated from seven iPBS markers

The un-weighted pair group method with arithmetic mean (UPGMA) dendrogram placed the 104 accessions into six clusters (Fig. 5), reflecting the PCoA results. The first cluster comprised five accessions of the related species *L. sibiricus*. The second cluster contained 10 accessions of *L. heterophyllus*. The third cluster included 10 accessions of *L. cardiaca* from A. The fourth cluster comprised 10 accessions of *L. cardiaca* from D. The fifth cluster included 15 accessions from S and 16 accessions from Kh. The sixth cluster represented 38 accessions including nine from S, seven from Kh, 14 from K and eight from T.

Discussion

Estimation of plant genetic diversity within and across geographic regions assists germplasm management

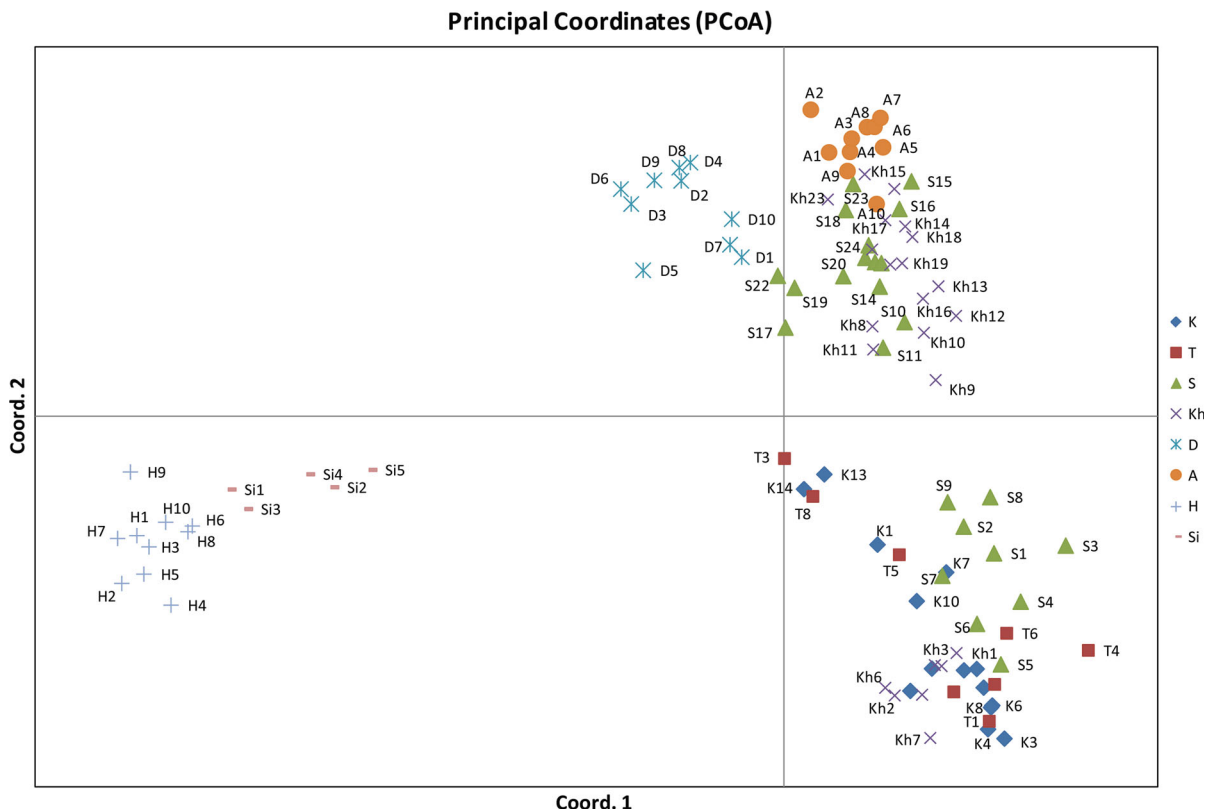


Fig. 4 Principal coordinate analysis of 104 motherwort accessions with seven iPBS primers

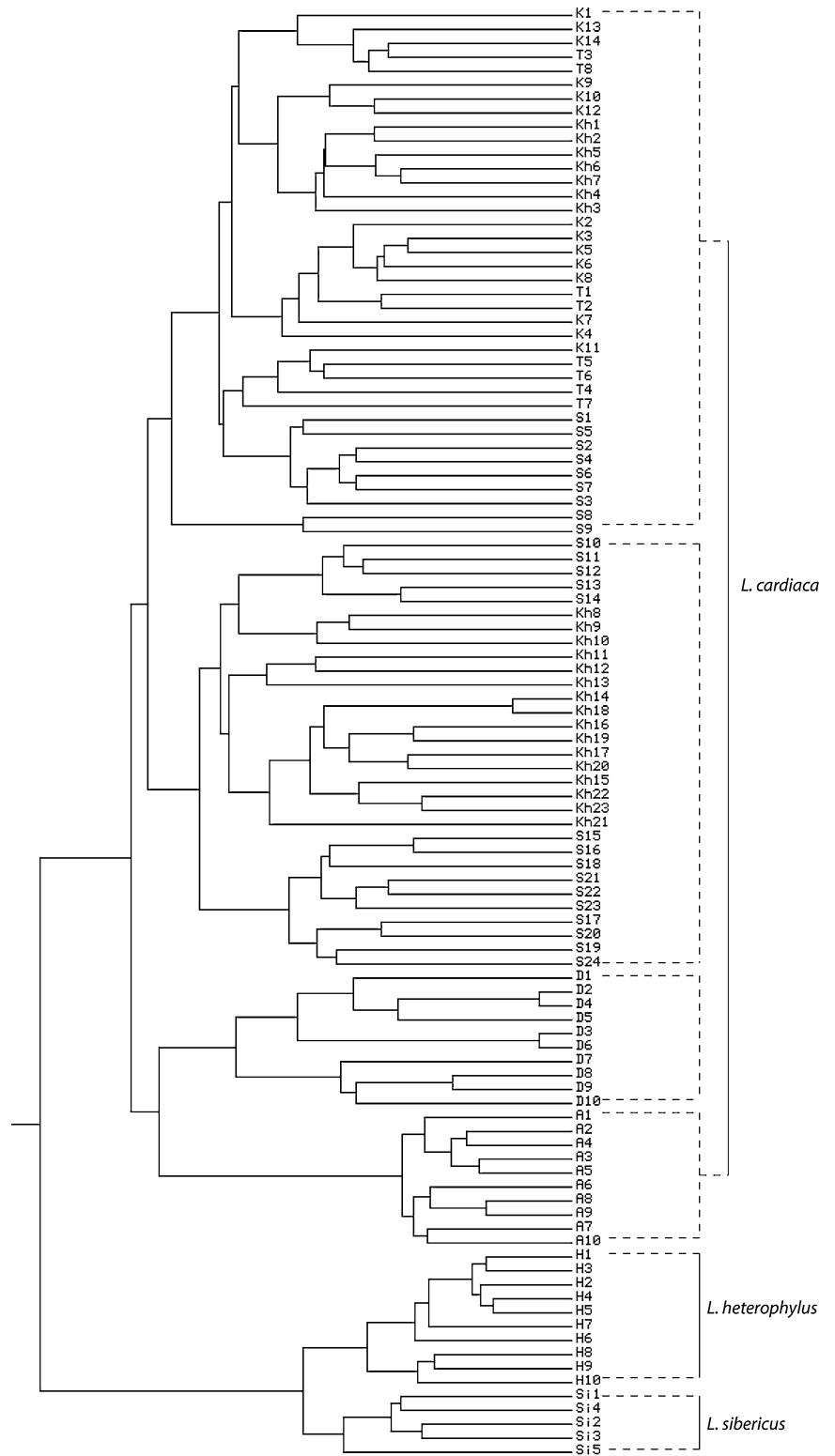
and conservation (Cardoso et al. 1998; Chalmers et al. 1992; Hamrick et al. 1991; Kim et al. 2005) and is an important first step in crop improvement (Mozaffarian 1996; Soorni et al. 2013a, b; Mehmood et al. 2014). Germplasm identification and the development of molecular marker systems such as the iPBS to assess genetic diversity and relationships enable targeted selection of parents independent of environmental influences. In our study, polymorphic iPBS markers enabled identification of a range of common motherwort (*L. cardiaca*) accessions and two related species of *L. heterophyllus* and *L. sibiricus*. This provided valuable information on the genetic relationships amongst these accessions.

The iPBS system differentiated accessions into separate groups within and across species and detected several species specific alleles. In particular, the species specific bands generated from primers 2390 and 2391 can be isolated for further detailed analysis. The information generated by the iPBS marker system suggests that this system can be used effectively for

diversity studies and genetic analysis in motherwort and related species. The relatively close genetic distances observed among the three motherwort species in this study also reflects their close genetic relationships in the natural environment. The species specific markers found in our study could then be aligned with phenotypic markers thus enabling population genetic analysis.

Similarly, genetic variation within and between populations of a particular plant species can be very important for breeding and conservation (Barrett and Kohn 1991; Ellstrand and Elam 1993). Population specific traits within the species *L. cardiaca* can also be used to optimize F_1 intraspecific hybridization. Our study revealed that intraspecific crosses among Iranian genotypes, specifically D/K and D/T, should generate more vigorous hybrids because of their greater genetic distances (0.10 or higher). All the intraspecific crosses between the Australian (A) population and the five Iranian populations should produce the most vigorous hybrids based on large genetic distances (0.106–0.136).

Fig. 5 Dendrogram of 104 Motherwort accessions generated with data from seven iPBS primers



0.1

The highest intraspecific genetic distance (0.136) exists between the Australian (A) population and the Dargaz (D) population from Iran. All the plants in population A have shiny leaves and a common ~1350 bp band generated from primer 2390. All the plants in population D are dwarf and carry a specific allele (~1300 bp) from primer 2391. Consequently, primers 2390 and 2391 and the two differentiating morphological traits could be used for selection of progeny from intraspecific crosses between populations A and D.

One hundred and ninety-one iPBS bands were obtained from seven primers, generating substantial polymorphism. The average number of bands for each primer were more than reported previously (Gailite and Rungis 2012; Guo et al. 2014; Baranek et al. 2012; Mehmood et al. 2013, 2015). The 12-mer primer 2391 produced the lowest number of bands (20) whereas primer 2390 generated the highest number of 32. Hence the length of the individual primer was not relevant to the number of bands amplified in this study.

The mean value of Shannon's Information index (0.213) in the current study is comparable to that reported by Khadivi-Khub and Soorni (2014). These authors reported average values of 0.26, 0.13, 0.13, 0.22 in Iranian materials when using AFLP, ISSR, RAPD and IRAP molecular markers, respectively. These relatively low values indicate significant inbreeding in the populations studied. Specifically, *L. cardiaca* accessions collected from any particular geographic area tended to have close genetic relationship thus indicating limited germplasm exchange or inter-population hybridization regionally. This result is comparable to studies by Popescu et al. (2009) and Heuberger et al. (2010) who reported that while *L. heterophyllus* was primarily cross-pollinated, it could self-fertilize in isolated environments. This infers that intraspecific hybridization between genetically distant populations of the motherwort genus *Leonurus* could enhance hybrid vigour and this could be subsequently captured through asexual propagation.

The characteristics of an effective molecular marker system are that it should be polymorphic in the materials tested and markers evenly distributed across the entire genome. It should distinguish genetic differences, be inexpensive to apply, rapid, require minimal amounts of DNA and require no previous knowledge of the genome (Alzohairy et al. 2014; Odong et al. 2011). The results of our study confirm that the iPBS method exhibits all these characteristics.

Other retrotransposon based molecular markers including sequence-specific amplified polymorphism (SSAP), retrotransposon-based insertion polymorphism (RBIP), inter retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellite amplified polymorphism (REMAP) could also be evaluated in the genus *Leonurus*, as outlined by (Alzohairy et al. 2014).

It is well-known that the botanical classification across the motherwort (*Leonurus*) genus suffers from a number of errors and it is a continuous task for botanists to eliminate such mistakes (Erdtman 1945; Wu and Li 1977). Heuberger et al. (2010) suggested that *L. heterophyllus* is genetically very close to *L. sibiricus* based on a range of morphological traits. The iPBS results from this study reaffirmed this position since the genetic distance found between these species was only 0.074. This indicates the potential power of the iPBS marker system to distinguish genetic diversity at the species level. Future research using the iPBS system could target other species within the *Leonurus* genus.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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