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Application of CAPS markers for diversity assessment in grass pea (*Lathyrus sativus* L.)

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Abstract. Genetic diversity among 20 *Lathyrus sativus* L. accessions from Ethiopia was investigated by using Cleaved Amplified Polymorphic Sequence (CAPS) markers. Genetic diversity statistics showed presence of a moderate level of genetic variation in the analysed accessions (P = 69.77%, Hs = 0.278). Analysis of genetic differentiation showed existence of a low level of differentiation between accessions, which accounted for only 7% of the total variation and most of the variation was due to differences among individuals within accessions (93%). Both cluster and principal coordinate analyses revealed minimal grouping of accessions based on geographical origin implying that local varieties of grass pea with different genetic backgrounds were distributed among various administrative regions in Ethiopia. The CAPS markers employed in our study demonstrated the utility of such markers for genetic diversity assessment in grass pea.

Key words: CAPS markers, genetic diversity, *Lathyrus sativus*

1. Introduction

Grass pea (*Lathyrus sativus* L.) is a legume crop used as human food and animal feed in different parts of the world, with specific importance in countries such as Ethiopia, India and Bangladesh (Campbell 1997). It is adapted to grow under extremely dry conditions, as well as on land subjected to flooding (Smartt *et al*. 1994). It can grow in a wide range of soil types with few agricultural inputs, and, when compared to other legumes, it has better resistance to many disease agents and pests (Campbell 1997). Its nutritional composition is comparable to other legume crops, such as field pea, which makes grass pea a good source of protein and starch (Vaz Patto *et al*. 2006). Grass pea is an important crop of economic significance for Ethiopia. Its ability to grow in harsh environmental conditions makes the crop preferable in many regions of the country where low input farming system is practiced. Currently it is cultivated on 9.6% of the total pulse production area of the country (CSA 2016).

Understanding the distribution and the structure of intraspecific diversity is essential for efficient conservation, management, and utilization of genetic resources. Different types of molecular tools are being used to evaluate genetic polymorphism in crop plants to achieve this purpose. However, with the exception of a few species, many legume crops have received little attention from advances made in molecular and genomic research (Varshney *et al*. 2007). This problem is also evident in crops like grass pea that are important for subsistence of local communities but do not have wider usage.

Polymorphic markers have been an important factor for diversity analysis and other genetic studies in various species. Among the molecular tools, diversity analysis on grass pea has been carried out by using RAPD (Croft *et al*. 1999), AFLP (Tavoletti & Iommarini 2007) and RFLP (Chtourou-Ghorbel *et al*. 2001). ISSR markers

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have been utilized to evaluate genetic relationships among different species in the Lathyrus genus (Belaid *et al*. 2006). Recently, however, progress has been made and additional molecular markers, which include EST-SSRs (Shiferaw *et al*. 2012; Sun *et al*. 2012) and genomic SSRs (Lioi & Galasso 2013; Yang *et al*. 2014; Wang *et al*. 2015) were developed to support genetic and genomic research on the species.

CAPSs (Cleaved Amplified Polymorphic Sequences) are markers, which are the result of restriction digestion of locus-specific PCR amplicons with appropriate restriction enzymes. Mutation in the restriction site creates or disrupts a restriction enzyme recognition site resulting in changes in the patterns of restriction fragments revealing polymorphisms (Konieczny & Ausubel 1993). CAPS markers have been successfully used for diversity analysis in different plant species (Tsumura & Tomaru 1999; Tsumura *et al*. 2007; Barth *et al*. 2002); variety identification (Hu *et al*. 2014), construction of linkage maps (Konovalov *et al*. 2005), and for marker assisted selection (Akashi *et al*. 2001; Gutierrez *et al*. 2006).

Publicly available Expressed Sequence Tags (ESTs) provide an excellent source for developing different types of DNA markers. These sources are useful especially for species whose genetic and genomic studies are very limited despite their potential and wider use in few countries. Sequence-derived markers have been published, which were developed by converting *L. sativus* defense-related ESTs into mapable genetic markers (Skiba *et al*. 2003), and some of these markers have been converted to CAPS markers. The objective of the present study is, hence, to evaluate the application of these markers for diversity studies in grass pea and to demonstrate the possibility of utilization of such markers for diversity assessment so that they can be exploited as alternative tools by themselves or in combination with other markers in species that lack adequate molecular tools.

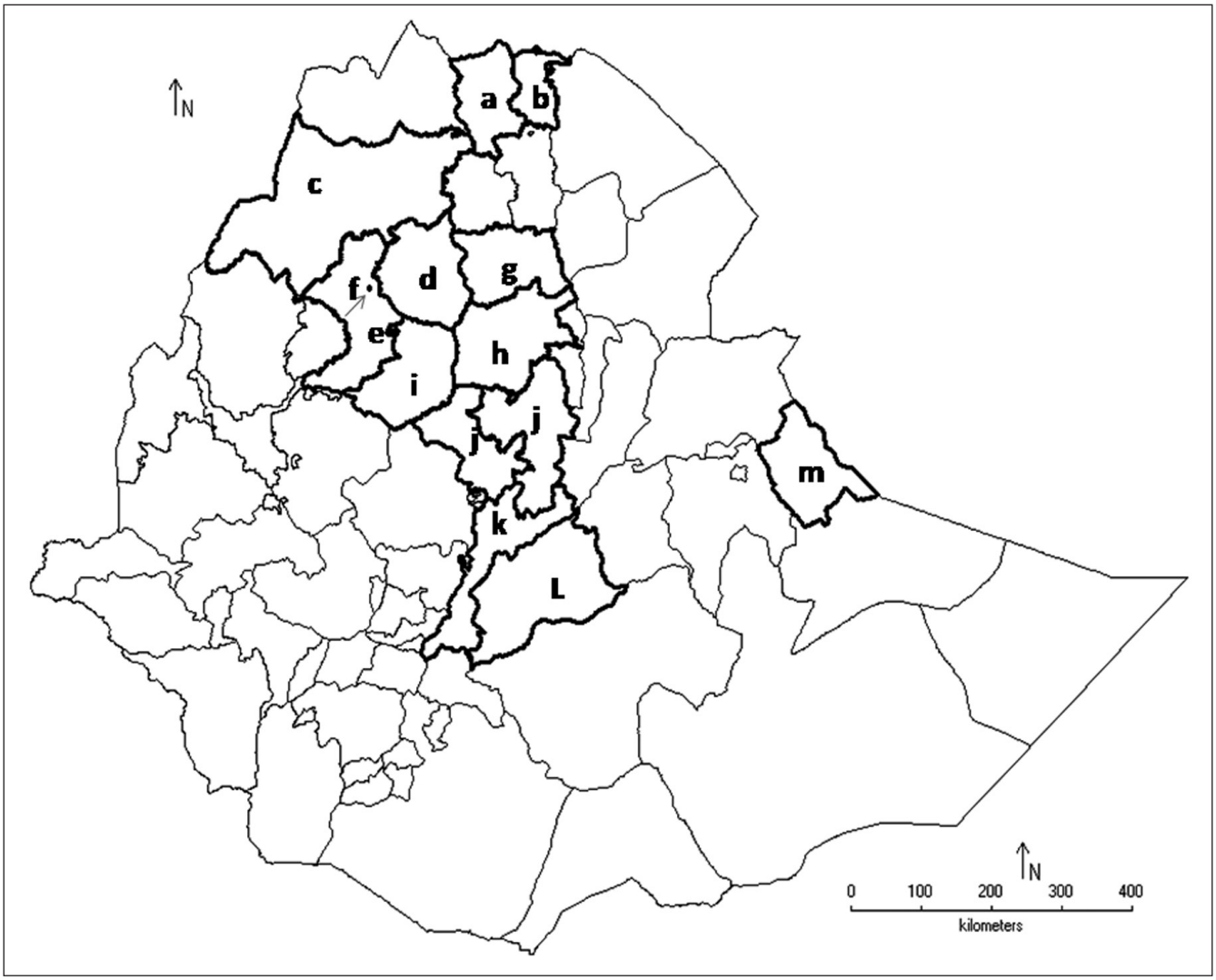

Fig. 1. Map of Ethiopia showing geographic origins of the grass pea accessions used in this study

Explanations: a – Central Tigray, b – East Tigray, c – North Gonder, d – South Gonder, e – West Gonder, f – Bahir Dar Sp., g – North Welo, h – South Welo, i – East Gojam, j – North Shewa, k – East Shewa, l – Arsi, M – Jijiga

2. Material and methods

2.1. Plant material

Twenty grass pea accessions were obtained from the genebank of Ethiopian Biodiversity Institute; EBI/ Ethiopia. They were chosen to represent different growing regions (Fig. 1). From each accession, twenty seeds were grown in a greenhouse and 15 randomly chosen individual plants were used for DNA extraction and diversity assessment.

2.2. DNA isolation and CAPS assay

Leaf samples were collected from two-week old grass pea seedlings. Genomic DNA was extracted with a GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO). DNA quality and quantification were estimated using 1% agarose gel electrophoresis and ethidium bromide staining, utilising lambda DNA as a reference.

We assayed 10 published STS primer pairs (Table 1). PCR amplification was performed in a 25 µL reaction volume containing: 5X PCR buffer, 0.2 mM each of dNTPs, 0.4 µM of forward and reverse primers, 1 unit of GoTaq® DNA polymerase (Promega), and 40 ng of genomic DNA. Cycle conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles with denaturing at 94°C for 45 s, annealing at optimal annealing temperature (varied with primers) for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Single monomorphic amplified products were digested with appropriate restriction endonucleases in a 10 µl total reaction volume. The reaction mix contained: 5 µL of PCR product, 1 µL restriction enzyme buffer, 1 unit restriction enzyme, and, when applicable, Bovine Serum Albumin (BSA) in a final concentration of 0.1 mg/mL. Amplicons and digested PCR products were resolved on 2% agarose gel in 1× TBE, stained with ethidium bromide and visualized with Gel Doc EQ™ (Bio-Rad). Consistency of results was assessed by analyzing random samples twice.

2.3. Data analysis

Results from the restriction digestion of the amplified products were transformed into binary data corresponding to the presence (1) or absence (0) of a specific fragment. The resulting binary data matrix

Table 1. Characteristics of CAPS markers tested and used for diversity assessment in grass pea

Primer name	NCBI acc. no.	Forward (F) and reverse (R) Primer sequence (5'-3')	T_a (°C)	Restriction Enzyme used	Size of digested PCR product (bp)	No. of polymorphic bands after digestion
159	DY396299	F: GCTTGAAGGGTTTTGATGGT R: ACAGAGGTTTATCGTCATTTTTCTC	58.3	*Hae* III	300	3
351	DY396332	F: GGGACCAAACAAAACCAAAA R: TCAGTAAGTAGCCAAGCCAATC	56	*Dde* I	1100	6
524	DY396349	F: GAGGGCCATTGTGCAAGT R: TCCCATTTAAGAGGCTTCACC	62.6	*Mnl* I	270	2
753	DY396385	F: CTGATGAGAAGTTCACTCGTTTG R: CTCCAGCACCAAATCCATAA	59	*Rsa* I	600	3
81	DY396281	F: GGTGACAAATACTGCAACTGG R: ACGAAATGATATGCCTTGTTTT	56.4	*Hae* III	250	2
1005	DY396430	F: ACCTTGTTCTCCCAGCTCTC R: GGCCAACTGCCTTATTCAAA	64	*Rsa* I	1100	6
2	DY396260	F: CTGAGCTGGTTGGTGTGA R: ATTGAAGGGAAAAGAAAAGACA	64	*ApoI, Mnl I*	320	NP
59	DY396276	F: CAAACACACATAGCATATTAAGTGAA R: CCATAAATGAGAAAGAAAATGGA	58.3	*Hinf I, Dde I*	600	NP
612	DY396358	F: AACCGCCGATGTGCT TTT R: TTTTCCCTTGGTGATTTTGG	54	*Rsa I, Hinf I*	560	NP
761	DY396389	F: GATGCTTCAGTGTTGTTTGGT R: ATACATTTTTATTTTATGGTAGATGCC	58.3	*Hinf I, Mnl I*	500	NP

Explanations: Ta-optimum annealing temperature, bp-base pair, NP-Not polymorphic

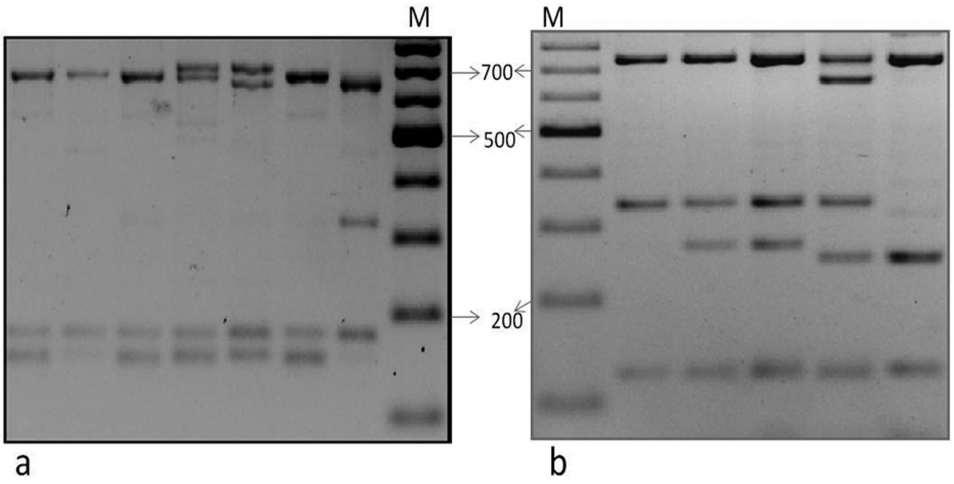

Fig. 2. CAPS polymorphism detected in grass pea accession using primer 1005 digested with *Rsa I* (a) and primer 351 digested with *Dde I* (b). M – Molecular weight marker (100bp)

for the 300 individuals was used to perform genetic diversity measures. Gene diversity was analysed by means of the Bayesian estimate of gene diversity, *hs*, using Hickory; Version 1.1 (Holsinger & Lewis 2006) using the default parameters; a burn-in period of 5,000 iterations, a run of 25,000 iterations and a thinning factor of 5. Each model was run five times to ascertain consistency and average values of the runs were used for model comparison. Genetic differentiation was determined using θ^{II}, a statistics similar to Nei's Gst. Percentage of polymorphic bands (Pb) and Principal Coordinate Analysis (PCoA) was performed using GenAlEx 6.5 (Peakall & Smouse, 2012). An unweighted pair group method with arthimetic mean (UPGMA) tree was produced with Nei's genetic distance using TFPGA version 1.3 (Miller 1997).

3. Results

Primers for our larger study were selected based on reproducibility and polymorphic patterns from initial screening on four accessions from different zones. Out of the 10 STS markers we assayed, six were polymorphic when digested with appropriate restriction enzymes (Fig. 2, Table 1), and they produced a total of 22 bands on the total analyzed samples.

3.1. Diversity and population structure

The DIC (Deviance Information Criterion), Dbar (measure of how well the model fits the data), Dhat and pD (approximate number of parameters being estimated) run in Hickory were interpreted following the recommendations given in the manual (Holsinger & Lewis 2006) to estimate the best fit model among the available models. The DIC and pD values were lower in the Full model than other models and this model was used for estimating gene diversity.

The average gene diversity estimates (hs) was 0.278 ± 0.005 (Table 2). The highest diversity was observed in accessions 7 and 14 from East Shewa and North Gondar zones, respectively, and the lowest variation was observed in accession 13 from the East Gojam zone. The percentage of polymorphic bands in the accessions averaged 69.77% and it ranged from 54.55% to 81.82%. Analysis of differentiation among accessions was done using the *free* model, which does not estimate f (the within-accession inbreeding coefficient). The most relevant statistics (θ^{II}), directly comparable to estimates of Fst, gave a mean differentiation value of 0.068 ± 0.014.

3.2. Cluster and Principal Coordinate analysis

The genetic relationship among the 20 grass pea accessions was analysed on the basis of the Nei (1972) genetic distance (Fig. 3). The result showed that, at similarity coefficient value of 0.5, the accessions were divided into five groups. Group I and IV contained one accession each, while the rest of the groups contained three to six accessions. Members of different accessions were grouped together and the overall clustering pattern did not follow the grouping of accessions according to their geographic origins. However, there were cases where accessions from the same origin were grouped together such as samples from Tigray (accession 5, 2 and 18). A close relationship between accessions from Tigray and the neighboring zone Gonder was also observed (group V).

Principal coordinate analysis (PCoA) on the basis of genetic distance was also used to visualize genetic association of accessions, which is primarily explained by the first two principal coordinates. The first and

Table 2. List of analysed accessions with geographic origin, percentage of polymorphic bands (Pb), and gene diversity (hs)

Accession code	Accession no.	Administrative Zone	hs	Pb (%)
1	46019	North Shewa	0.276	63.64
2	234043	Central Tigray	0.288	68.18
3	236700	Bahir Dar, Gojam	0.272	54.55
4	235018	North Welo	0.275	63.64
5	238241	Central Tigray	0.287	72.73
6	238945	West Gojam	0.291	72.73
7	238955	East Shewa	0.295	77.27
8	236562	East Shewa	0.293	81.82
9	231325	Arsi	0.272	63.64
10	238931	South Gonder	0.262	68.18
11	236568	North Shewa	0.260	59.09
12	46106	Welo	0.278	77.27
13	46015	East Gojam	0.259	59.09
14	238929	North Gonder	0.295	81.82
15	215706	South Welo	0.281	72.73
16	46050	East Gojam	0.272	72.73
17	215246	South Welo	0.278	81.82
18	207497	East Tigray	0.279	77.27
19	212742	South Gonder	0.277	72.73
20	241143	Jijiga, Harerge	0.265	54.55
Mean			0.278±0.005	69.77±1.96

second coordinates extracted 20.06% and 15.23% of the total molecular variation, respectively. The individual samples belonging to different accessions formed no distinct groups based on accessions. Similar to the cluster analysis, PCoA also showed no clear separation of the populations based on geographic origins (Fig. 4).

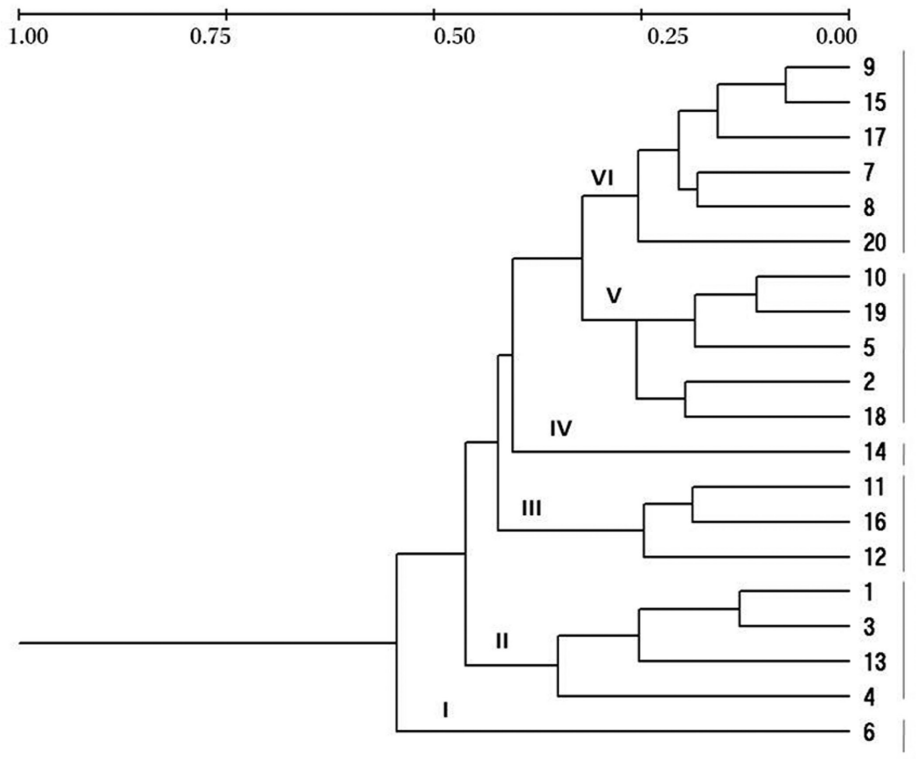

Fig. 3. Unweighted pair group with arithmetic mean (UPGMA) dendrogram based on Nei's genetic distance between 20 grass pea accessions with six CAPS markers

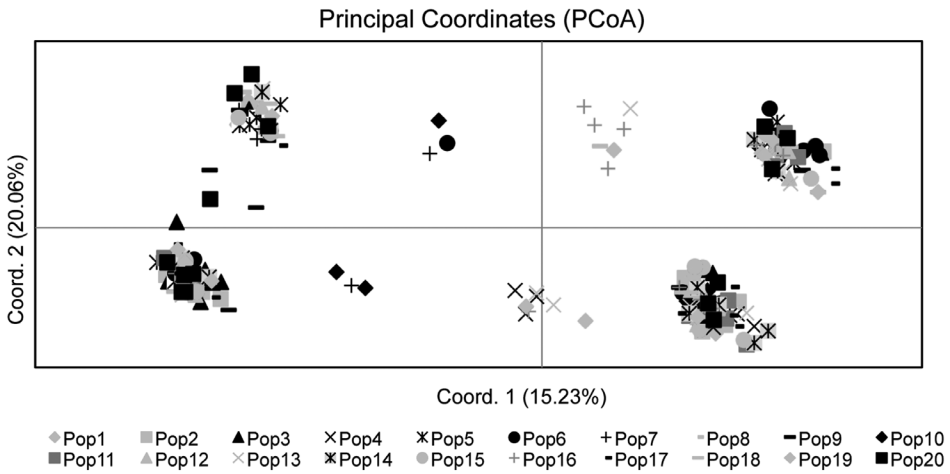

Fig. 4. Association among 20 accessions (populations 1-20) of grass pea revealed by principal coordinate analysis

4. Discussion

CAPS markers, developed by converting *L. sativus* ESTs into genetic markers by Skiba *et al*. 2003, were used for diversity assessment in accessions of the same species. The mean gene diversity level (hs=0.278) indicates presence of a moderate level of diversity among the analysed accessions. Previous studies on grass pea accessions from Ethiopia reported presence of significant variation using morphological markers (Tadesse & Bekele 2003), while Chowdhury & Slinkard (2000) reported a relatively lower level of diversity among grass pea accession collected from Ethiopian region by using isozyme markers. Seven *L. Sativus* based EST-SSR markers resulted in a total of 25 alleles and the six CAPS markers used in this study resulted in 22 polymorphic bands after restriction digestion. EST-SSR markers revealed a higher level of diversity (He=0.419) on Ethiopian accessions (Shiferaw *et al*. 2012), which shows a better discriminating capacity of these markers than the CAPS markers. However, the level of polymorphism and gene diversity observed using CAPS markers show that these markers are also informative tools that can be used independently or in combination with other types of markers.

Cluster analysis and PCoA resulted in the grouping of accessions and individual plants irrespective of their region of origin implying that local varieties of grass pea with different genetic backgrounds were distributed across various geographic regions. On the other hand, there were cases where samples from the same or neighboring zones occurred in the same cluster which could imply that material exchange was stronger among neighbouring regions than among those distantly apart. Grouping of grass pea accessions from different origins in the same group was also previously reported using morphological markers (Tadesse & Bekele 2003).

Breeding system, gene flow, isolation mechanisms and intensive selection by natural and experimental pressures can influence the genetic structure of a population (Chandel & Joshi 1983). In our study, the f = 0 model could be considered as likely as the Full model, since the difference in Dbar values between these two models was low (0.59), hence inbreeding is an unlikely driving force in determining the structure in the studied population (Holsinger & Lewis 2006). The low level of differentiation among accessions, which accounted for only 7% of the total variation observed here, could result from seed exchange among farmers and human relocation. It could also be attributed to the type of marker used since EST-SSRs showed higher differentiation (F_{ST} = 0.15, P< 0.001) value (Shiferaw *et al*. 2012).

To our knowledge, this is the first attempt to apply CAPS markers for diversity assessment in *L. sativus*. The set of CAPS markers employed in this study demonstrates the utility of such markers for genetic diversity analysis. Similar markers can be developed from newly developed DNA sequences for the species (Lioi & Galasso 2013; Yang *et al*. 2014) available in the public database. These markers could be useful for examining patterns of genetic diversity in grass pea accessions across a wider scope of geographical locations. The requirement of relatively cheap equipment and procedures, which include PCR, digestion with restriction enzymes and simple identification of fragments after digestion resolved by agarose gel electrophoresis is an advantageous property of CAPS markers. They can be exploited as alternative molecular markers either individually or in combination with other marker types in species that lack sufficient molecular tools.

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