

First use of microsatellite markers in a large collection of cultivated and wild accessions of tepary bean (*Phaseolus acutifolius* A. Gray)

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Received: 20 December 2011 / Accepted: 18 May 2012 / Published online: 8 June 2012
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Abstract Tepary bean (*Phaseolus acutifolius* A. Gray) is a dry-land crop species that originated in the deserts of Mexico and the south-western United States and therefore is proposed as a source of drought and salt tolerance for related species and for production in marginal rainfall areas. Few genetic tools have been developed or tested for tepary bean but microsatellites from common bean are an obvious choice for diversity analysis in the crop. The first goal of this study was to validate a set of gene-derived and non-gene simple sequence repeat or microsatellite markers from common bean in tepary bean cultivars and wild relative accessions. The second and more extensive objective of this study was to evaluate the genetic diversity and population structure of the tepary bean accessions to determine if leaf-morphology variants are valid as separate sub-groups of wild tepary beans; if *P. parvifolius* exist as a separate variants or species; and if cultivated tepary beans originated from one domestication event or several events. Our analysis of 140 tepary bean genotypes showed that a single domestication was likely as the cultivars were most closely related to accessions from Sinaloa and northern Mexico and that diversity was much higher in the wild genotypes compared to the cultivated ones. Other results were that *P. parvifolius* was classified as a separate species by population structure analysis while the variants *P. acutifolius* var. *acutifolius* and var. *tenuifolius* were

admixed and inter-crossed. *P. latifolius* is not a valid species or variant of *P. acutifolius* but represents a group of cultivars within tepary bean. This is the first analysis of microsatellite diversity in tepary beans and has implications for breeding and conservation of this crop and its wild relatives.

Introduction

Tepary bean (*P. acutifolius* A. Gray) is a drought-tolerant but neglected crop of Middle America, the region between North and Central America (Blair et al. 2002). The species and crop were thought to have originated and been domesticated in the region from Central Mexico to Southwestern United States (Freeman 1912, 1913; Carter 1945; Nabhan and Felger 1978; Manshardt and Waines 1983). However, the specific center of origin is not confirmed. Tepary bean is part of the suite of crop plants from Mesoamerica (Vavilov 1931) that were used at the drier northern borders of this region. Tepary beans fit into a different agroecology than other five *Phaseolus* species that were domesticated in more humid regions of Central America, the Andes and the Amazon basin.

The present geographical distribution of the cultivated form of tepary bean extends in the north from Arizona and New Mexico in the United States to Guanacaste province in Costa Rica in the south. Within this region, tepary beans are found on the dry subtropical slopes of the Pacific, not on the more humid Atlantic coastal plains (Debouck 1991). From this distribution, one can observe that the present range of the crop is mostly limited to its center of domestication with little to no adoption in other regions of the Americas or the world for that case. This is in contrast to the other cultivated species of the genus *Phaseolus*,

Communicated by B. Diers.

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where *P. vulgaris* L. (common bean), *P. coccineus* L. (runner bean), *P. dumosus* Macfady (previously *P. polyanthus* or year-long bean) and *P. lunatus* L. (Lima bean), which have all been adopted to greater or lesser degree outside their centers of origin (Debouck 1991; Freytag and Debouck 2002).

Renewed interest in tepary bean has been due to its potential to adapt to climate change and the fact that a desert species like *P. acutifolius* is tolerant to drought and salty soils. It is also early maturing and high yielding. This interest in tepary bean has come from two fronts: one for the direct use of the species as a quick and easy-to-grow crop for low rainfall (≤ 250 mm per year) areas in Peru, India or the northern Sahel and second for transfer of drought-tolerance genes to common bean (Blair et al. 2002; Singh et al. 1998). Drought tolerance in tepary bean is due to differential gene expression and to a different morphology and phenology compared to common bean (Freytag and Debouck 2002; Micheletto et al. 2007). Earliness to flower as well as small, waxy leaves are typical of most varieties of tepary bean.

In addition, tepary bean has been the source of resistances to several diseases that affect common bean. These include common bacterial blight caused by the pathogen *Xanthomonas axonopodis* pv. *phaseoli* (Coyne et al. 1963), ashy stem blight caused by *Macrophomina phaseolicola* (Miklas et al. 1998) and bean rust caused by *Uromyces appendiculatus* (Urrea et al. submitted). It is generally accepted that some disease resistances have been transferred successfully by crossing tepary and common beans while drought tolerance has not. Furthermore, tepary bean is the only species of the *Phaseolus* group of cultigens that can be regularly transformed with *Agrobacterium tumefaciens* (Zambre et al. 2005) and transgene transfer into tepary beans is another reason for interest in the crop.

Finally, tepary bean is part of the tertiary gene pool of common bean and can be crossed with this important species using the congruity backcross method (Muñoz et al. 2004) obtaining some introgression of genes from one species to the other especially for the breeding line XAN159 (Muñoz et al. 2004). Some genomic resources have been developed for common bean genotypes that have tepary bean genomic introgressions, but few molecular tools have been specifically developed for or tested on tepary bean. It is worth noting that cultivated tepary bean can be crossed easily with several wild relatives within the species or in other species such as *P. parvifolius* increasing the chance for polymorphic marker analysis of genetic traits of interest (Blair et al. 2003b).

In this study, our focus was on the use of common bean microsatellites to evaluate diversity in tepary bean

genotypes from both the cultivated and wild gene pools as well as from related species. Interestingly, the wild tepary beans appear to be more diverse in leaf morphology than the cultivated types (Debouck 1999). However, controversy still exists on the number of domestication events for the crop with some authors proposing two domestications in the northern and southern parts of the range (Manshardt and Waines 1983). Other authors suggest a single origin but different locations for domestication either in the Mexican state of Durango (Schinkel and Gepts 1988) or the states of Sinaloa or Jalisco (Garvin and Weeden 1994), all of this work being based on phaseolin or isozyme analysis. The source of domesticates is most likely in wild tepary bean accessions of *P. acutifolius*, for which two varieties exist: *P. acutifolius* var. *acutifolius* and *P. acutifolius* var. *tenuifolius* the latter with even narrower leaflets than the first.

In previous work from our laboratory (Muñoz et al. 2006), we predicted like Garvin and Weeden (1994) that domestication might have been from wild genotypes of *P. acutifolius* var. *acutifolius* based on amplified fragment length polymorphism (AFLP) markers, but could not rule out multiple domestications. Furthermore, a controversy exists on one set of tepary beans given that all cultivated tepary beans are known to be *P. acutifolius* but some with wide leaflets have been termed *P. latifolius*, a species which is not widely accepted by researchers. Finally the relationship of *P. acutifolius* with other species within the section *acutifolii* of the genus *Phaseolus* is of interest as crosses can be made between cultivated tepary beans and a related species *P. parvifolius* (Blair et al. 2003b).

The specific objectives of this work, therefore, were (1) to confirm that a group of diagnostic microsatellite markers from common bean can amplify in a range of tepary bean accessions, (2) to evaluate diversity within the cultivated and close relatives of tepary bean using microsatellite markers and (3) to help determine where domestication of the crop took place. We evaluate a total of 140 wild and cultivated tepary including a large group of germplasm based on cultivated *P. acutifolius* or wild *P. acutifolius* var. *acutifolius*, *P. acutifolius* var. *tenuifolius* and *P. parvifolius* accessions to determine diversity levels among and between different subgroups of leaf-variant accessions. For diversity analysis, we used a set of high-polymorphism microsatellite markers from common bean which have proven useful for race evaluation (Díaz and Blair 2006; Blair et al. 2007). Microsatellite markers from common bean were known to be transferable to other species of *Phaseolus* (Gaitán-Solís et al. 2002; Blair et al. 2002, 2006) but have not been widely tested in a range of *P. acutifolius* and wild relatives.

Materials and methods

Plant materials

A total of 140 tepary bean and related wild genotypes were analyzed in two groups. The first group consisted in 39 representatives of different mostly wild relatives of tepary bean including the wild species *P. parvifolius* and all the variants of wild *P. acutifolius* with a few cultivars and was used to determine relationships between these groups. The second group consisted of 101 cultivated and wild tepary bean genotypes from the species *P. acutifolius* (cultivars, var. *acutifolius* and var. *tenuifolius*) and from *P. parvifolius* and was focused on analyzing the possible source or sources of the domesticated cultivars in wild populations of the two variants. These accessions are all part of the FAO (Food and Agriculture Organization) collection held in trust by the Genetic Resources Unit of CIAT (International Center for Tropical Agriculture). Of the 140 tepary beans and wild relatives analyzed, 57 consisted in cultivated *P. acutifolius* var. *acutifolius*, 37 in wild *P. acutifolius* var. *acutifolius*, 27 in *P. acutifolius* var. *tenuifolius*, 7 in *P. parvifolius* accessions, 4 in *P. acutifolius* var. *latifolius* and 8 in an outgroup of other *Phaseolus* species representatives (*P. coccineus*, *P. lunatus*, and *P. vulgaris*). Complete information on the genotypes and the geographic origin of each accession can be found in Table 1 of Muñoz et al. (2006). The genotypes were grown in the greenhouse and total genomic DNA was obtained from 2 g of fresh leaf tissue with a CTAB-based extraction method (Afanador et al. 1993).

Microsatellite genotyping

A total of 20 microsatellites were amplified for the study; of which ten were genomic (BM114, BM172, BM181, BM183, BM197, BM201, BMd11, BMd12 and BMd36) and ten were cDNA based (BMd1, BMd7, BMd10, BMd15, BMc121, BMc123, BMc124, PV-ctt001 and PV-at003). More information on these microsatellite markers is available in the publications of Gaitán-Solis (2002) for BM markers, Blair et al. (2006) for BMd markers, Blair et al. (2009) for BMc markers and Yu et al. (2000) for PV markers. In all cases the PCR amplification, profiles and reactions recommended by these previous authors for these molecular markers were used. All PCR products were run on 4 % silver-stained polyacrylamide gels and the alleles were sized by comparison with 10 and 25-bp molecular weight ladders. Alleles were considered separate taxonomic units for the purposes of calculating shared bands and similarity.

Data analysis

Genetic similarities between genotypes in each group were determined with a coefficient based on the proportion of shared alleles and on a principal component analysis (PCoA) using the software package SAS (Statistical Analysis Systems, Cary, North Carolina). Dendrograms were then built for the first and second set of genotypes using Darwin v. 5.0 software (Perrier et al. 2003) and the simple matching coefficient with the neighbor joining algorithm for each group. So as to compare with results from Muñoz et al. (2006), we also conducted UPGMA clustering for all the genotypes including the outgroup species using a Euclidean distance coefficient from the PCoA. Then, population structure was determined using the software program STRUCTURE (Pritchard et al. 2000) varying the *K* values (population number) from 2 to 5. Totals of 50,000 burn-ins and 100,000 replicates along with 15 independent simulations were used for population structure analysis. Finally, PowerMarker v. 3.25 (Liu and Muse 2005) was used to evaluate the number of alleles.

Results

Allelic diversity in the tepary bean sample

Our first results were that all 20 common bean microsatellite amplified DNA from tepary bean species. The 20 microsatellites detected a total of 165 alleles which were scored as bands to determine genetic similarity between genotypes. Of the total number of alleles, 81 were based on genic microsatellites and 84 alleles were based on genomic microsatellites. The average number of alleles per locus across the range of genotypes was almost as high for the cDNA (8.1 alleles) as for the genomic (8.4 alleles) microsatellites and overall the average was 8.25 alleles. The range of alleles per locus was from 4 to 16 for gene-based microsatellites and 5 to 16 for genomic microsatellites. Expected allele sizes were similar in gene and non-gene derived markers as shown in Table 1 and the markers were distributed over nine chromosomes based on map positions in common bean. The individual microsatellite loci with the greatest numbers of alleles were BM172, BM183, BMd1, BMc121 and PV-at003 all having 10 or more alleles.

Grouping of wild and cultivated tepary beans

Neighbor joining (NJ) analyses based on the microsatellite allele patterns were useful in distinguishing groups of tepary beans in both the first and second sets of genotypes (Figs. 1 and 2, respectively). In the first set of genotypes,

Table 1 Characteristics and number of alleles detected by the gene-based and genomic microsatellites evaluated on wild and cultivated tepary beans

SSR evaluated	Forward primer	Reverse primer	LG	Exp. size	Number of alleles
Gene-based					
BMd1	CAAATCGCAACACCTCACAA	GTCGGAGCCATCATCTGTTT	B03	165	11
BMd7	GGATATGGTGGTGATCAAGGA	CATACCCAATGCCATGTTCTC	B02	166	6
BMd10	GCTCACGTACGAGTTGAATCTCAG	ATCTGAGAGCAGCGACATGGTAG	B10	139	7
BMd15	TTGCCATCGTTGCTTAATTG	TTGGAGGAAGCCATGTATGC	B04	166	5
BMd53	TGCTGACCAAGGAAATTCAG	GGAGGAGGCTTAAGCACAAA	B05	105	4
BMc121	TGCATTCACCGCTATTACGA	CACTGTAGCCACCATGAGCA	NA	160	16
BMc123	CCTCCACCTTCTCCCTTC	TTACCATTCAATGGTTTATTAGCA	NA	164	6
BMc124	TGTCGGTTGTGAGACAGGA	TTGGAGCTGCTACTCCCACT	NA	122	8
PV-ctt001	GAGGGTGTTCCTACTATTGTCCT	TTCATGGATGGTGGAGGAACAG	B04	152	8
PV-at003	ACCTAGAGCCTAATCCTTCTGCG	GAATGTGAATATCAGAAAGCAAA	B04	139	10
Sub-Total (Average)	–	–	NA	(147.8)	81 (8.1)
Genomic					
BMd11	GCTCAACATTCCAGAGGCTAA	TCAAACCTACATAAATAAAACAAAACA	NA	161	7
BMd12	CATCAACAAGGACAGCCTCA	GCAGCTGGCGGGTAAAAACAG	B06	167	5
BMd36	CATAACATCGAAGCCTCACAGT	ACGTGCGTACGAATACTCAGTC	B03	164	9
BM114	AGCCTGGTGAATGCTCATAG	CATGCTTGTGCTTAECTCTCT	B09	234	7
BM172	CTGTAGCTCAAACAGGGCACT	GCAATACCGCCATGAGAGAT	B03	107	10
BM175	CAACAGTTAAAGGTCGTCAAATT	CCACTCTTAGCATCAACTGGA	B05	170	9
BM181	ATGCTGCGAGTTAATGATCG	TGAGGAGCAAACAGATGAGG	B03	192	5
BM183	CTCAAATCTATTCACTGGTCAGC	TCTTACAGCCTTGCAGACATC	B07	149	16
BM197	TGGACTGGTGCATACGAAGC	CCCAGAAGATTGAGAACACCAC	B03	201	7
BM201	TGGTGTACAGACTTGATGG	TGTCACCTCTCTCCTCCAAT	B07	102	9
Sub-Total (Average)	–	–	NA	(147.7)	84 (8.4)
Overall Total (Average)	–	–	NA	(147.75)	165 (8.25)

LG linkage group location in common bean for the microsatellites based on Blair et al. (2003a, b) along with expected (Exp.) size, SSR simple sequence repeat or microsatellite

the subgroups generally agreed with the grouping based on varietal classes and species for wild relatives of tepary beans: *P. acutifolius* var. *acutifolius*, *P. acutifolius* var. *tenuifolius*, *P. acutifolius* var. *latifolius* and *P. parvifolius*. In the dendrogram for this group (Fig. 1), all the *P. parvifolius* accessions grouped together except for G40080, an accession from Chihuahua, which was closer to *P. acutifolius* var. *tenuifolius*. The accessions of *P. acutifolius* var. *tenuifolius* also grouped together but in six sub-clusters. These clusters consisted in first G40093 and G40101 from Durango and Sinaloa; second, five accessions all from Durango (G40087, G40095, G40106, G40178 and G40239); third, four accession all from Arizona (G40240, G40261, G40263 and G40273); fifth G40071 and G40114 also from Arizona; and sixth, two accessions from Jalisco (G40104 and G40105).

Apart from these sub-groups, one branch of *P. acutifolius* var. *tenuifolius* (consisting of six accessions G40104, G40106, G40210, G40211, G40217 and G40233) grouped with the wild *P. acutifolius* var. *acutifolius* accession from Texas: G40107. Meanwhile, all the *P. latifolius* accessions (G40177B, C, D and E) grouped closely together and within a branch of cultivated *P. acutifolius* including G40089, G40096 and G40286, showing their similar origin and close relationship with tepary beans. Finally, the wild accessions of *P. acutifolius* var. *acutifolius* (G40197, G40199, G40236, G40242 and G40244) two of which were from Durango, two from Zacatecas and one from Michoacan, also grouped together. No exact duplicates were detected in this analysis but grouping of sub-accessions and accessions from the same collection site and consecutive collection numbers showed the precision of microsatellite genotyping.

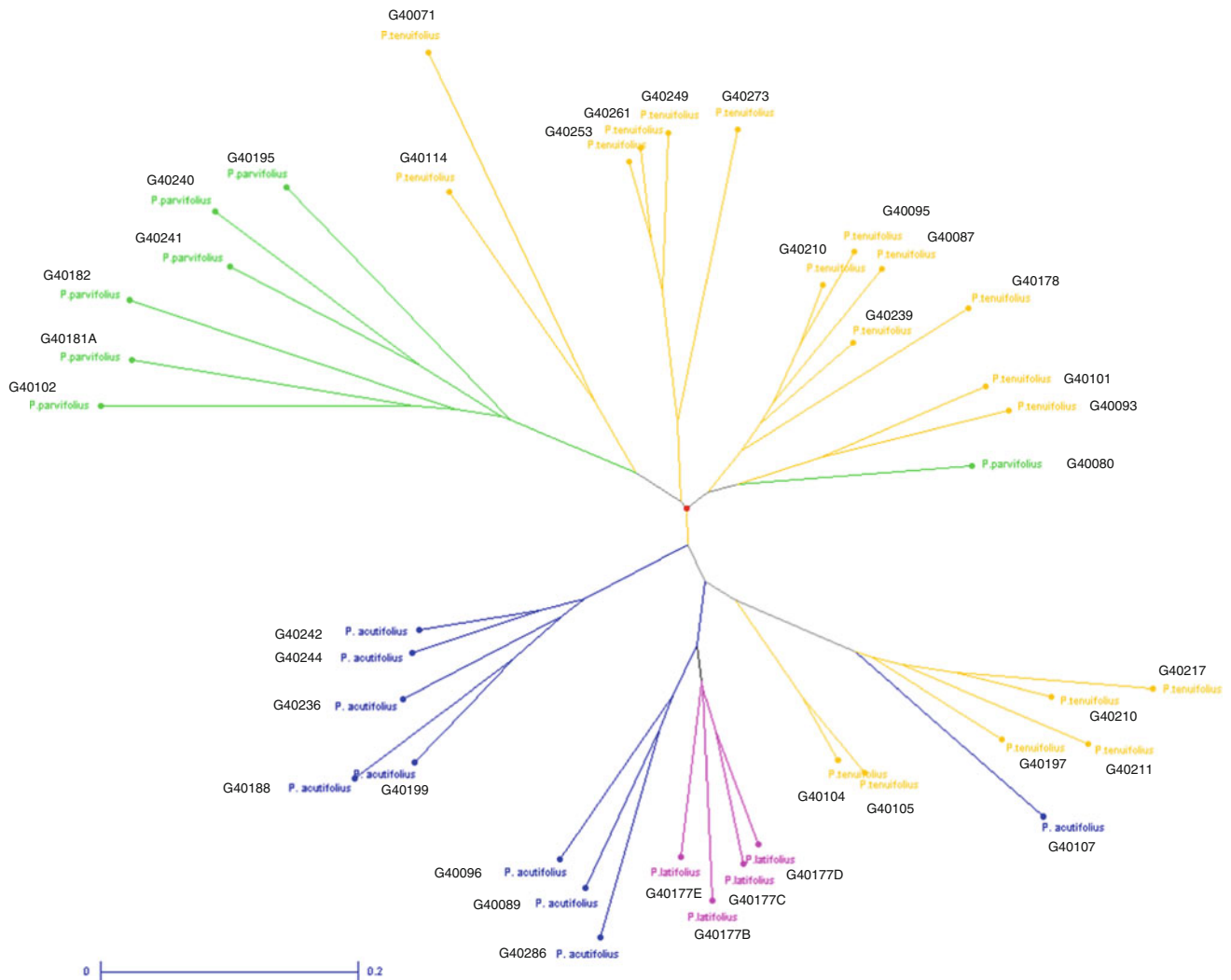


Fig. 1 Neighbor joining dendrogram showing the relationships among *P. acutifolius* (blue lines) and wild relatives *P. acutifolius* var. *tenuifolius* (yellow lines) and *P. parvifolius* (green lines). Non-standard species *P. latifolius* (a.k.a cultivated *P. acutifolius*) is

indicated as well (purple lines). Accessions are indicated with their “G” number as explained in the text and scale represents proportion of shared alleles (color figure online)

A similar dendrogram for the second group of mixed wild and cultivated genotypes (Fig. 2) showed the high similarity of the cultivated tepary beans compared to the wide diversity found in the wild tepary beans. We also found evidence for a single domestication event in Sinaloa or Sonora where wild tepary beans were most similar to the cultivated types. In this dendrogram, the cultivated genotypes were all located tightly clustered in the center of a bifurcated tree. From this central cluster, one branch included wild *P. acutifolius* var. *acutifolius* and the other branch included some of the same wilds in addition all the other variants of tepary bean and *P. parvifolius* accessions. One accession reported as cultivated (G40272) grouped with the wild accessions of tepary bean and probably was a misidentified genotype or a wild accession found in a cultivated field.

Some of the cultivated genotypes had almost exactly the same fingerprints and could be studied further as possible duplicates. Meanwhile, none of the wild accessions were exactly the same; although G40081 and G40121 were two *P. acutifolius* var. *tenuifolius* accessions that were highly similar within a cluster of related accessions. The separation of *P. acutifolius* var. *acutifolius* and *P. acutifolius* var. *tenuifolius* was less clear in this large analysis compared to the first group of selected genotypes. Finally, the *P. parvifolius* accessions were all distributed at the end of the dendrogram and were most distant from cultivated tepary bean and the Sonoran-Sinaloan accessions that we propose gave origin to the cultivars. Among the accessions of the distinct species, G40183, G40184 and G40185 were closely related. Similarly, G40170 and G40268, two *P. parvifolius* accessions from Jalisco in Mexico; and G40075

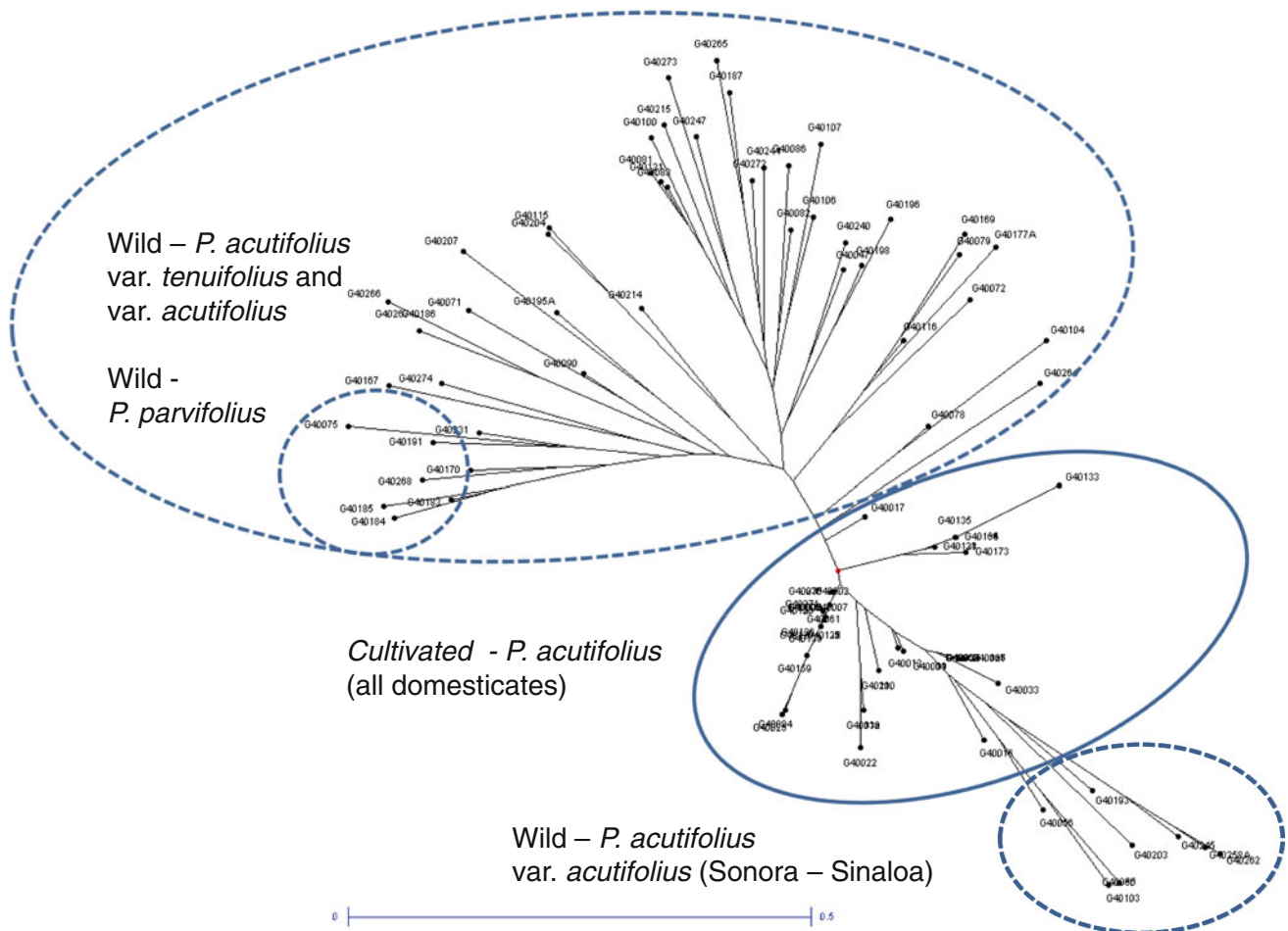


Fig. 2 Neighbor joining dendrogram showing the relationships (circles) among cultivated tepary bean (*P. acutifolius*) at the center and wild variants of *P. parvifolius* or *P. acutifolius* var. *acutifolius* and var. *tenuifolius* at the extremes. The accessions of Sonoran-Sinaloan *P. acutifolius* var. *acutifolius* that are most closely related to

cultivated tepary bean are shown in the lower right hand corner while the more distant accessions of *P. parvifolius* or *P. acutifolius* var. *acutifolius* and var. *tenuifolius* are in the large circle to the upper left hand corner. Scale represents proportion of shared alleles

and G40191, two *P. parvifolius* accession from New Mexico in the United States, were related.

Population structure of tepary beans

The STRUCTURE analysis confirmed sub-group divisions observed in the first and second group of genotypes. In the population structure analysis we show the divisions at different K -levels (where K = the number of sub-populations) for the cultivated and wild relative tepary beans. We decided not to include the outgroup genotypes or other species in the population structure analysis as these genetic differences are already known and well-established. Figure 3a and b shows the STRUCTURE results for each of the genotype groups, respectively. In both figures, the different colors indicate inclusion in separate sub-populations. The vertical bars represent each genotype while

division of the bars shows the genome content based on the sub-population identity.

In the first group of genotypes (Fig. 3a), the division at $K = 4$ was shown to be the mostly descriptive based on an Evano test and biological interpretation. Here, we found the separation of accessions of *P. acutifolius* var. *tenuifolius* in the upper branch of the dendrogram admixed with a group of *P. acutifolius* var. *acutifolius* accessions from the same branch. Next, a group of *P. acutifolius*, *P. acutifolius* var. *acutifolius* and *P. latifolius* accessions was found all mixed together but divided from the *P. parvifolius* accessions as a separate sub-group with no admixture between this species and the other. Separation of the population into other K -values was less significant in this analysis and was not considered further.

Meanwhile, in the second group of genotypes (Fig. 3b) we found that division at $K = 2$ was most significant. In

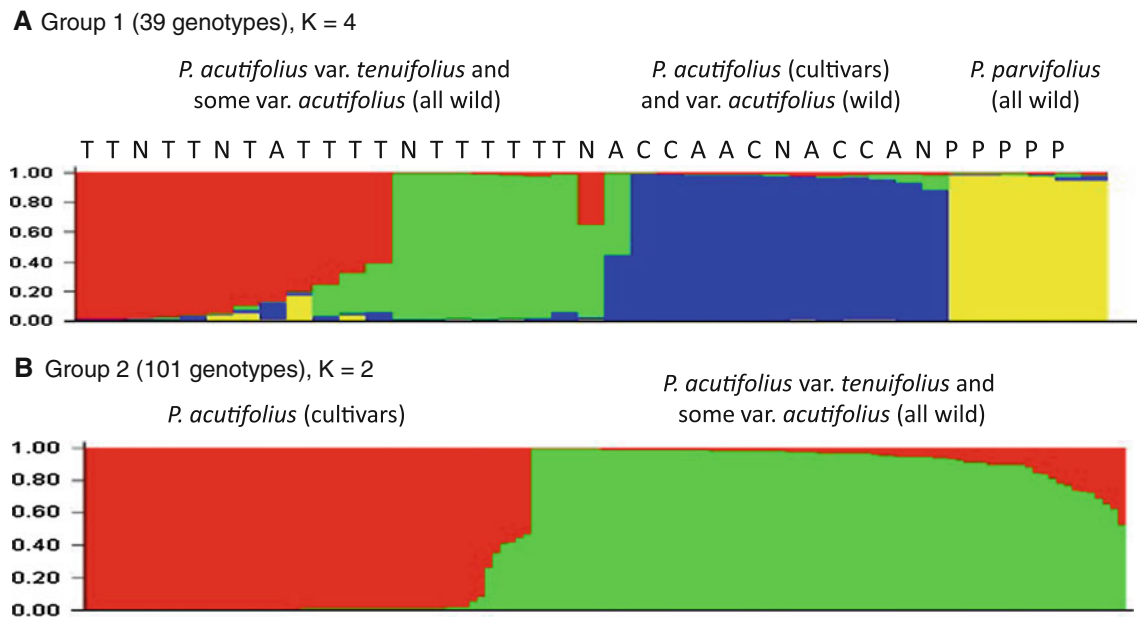


Fig. 3 Populations structure analyses for **a** the first and **b** the second group genotypes at $K = 4$ and $K = 2$ sub-population numbers, respectively, as described in the text. Sub-population identities are indicated above the graphs

this analysis, all but two of the cultivated tepary bean genotypes (*P. acutifolius*) were separate from all the wild relatives, which were not clearly separated into sub-populations. The cultivars of tepary bean showed little diversity or admixture while the wild accession group was very diverse and mixed in terms of being *P. acutifolius* var. *acutifolius*, *P. acutifolius* var. *tenuifolius* and *P. parvifolius*.

Discussion

Cross species amplification of microsatellites

In this research, we found that all of the common bean-derived microsatellites tested were useful in the analysis of tepary beans. This is interesting since the microsatellites were from various sources, including both gene-derived and non-genic microsatellites (Blair et al. 2003a, 2006). The BMC and PV markers from Blair et al. (2009) and Yu et al. (2000), respectively, had not been tested on tepary bean DNA before but were shown to be amplifiable in the PCR for this species.

These results confirm the findings of Gaitán-Solis et al. (2002) for non-gene based SSRs and extend the cross-species amplification to gene-based microsatellites from Yu et al. (2000) and Blair et al. (2003a, 2006). We expected genic microsatellites to amplify well even across species boundaries and genomic microsatellites seem also to amplify in tepary bean accessions. The set of microsatellites used was complementary in that various sources

of markers were considered and these have been genetically mapped in common bean to different chromosomes (Blair et al. 2009). It would be interesting to screen the full set of microsatellites available in common bean (above 2000) for amplification in tepary bean and wild relatives. For example, the gene-based FJ markers from Hanai et al. (2010) might also be functional in tepary bean. The same sort of analysis could be conducted for *P. lunatus*, the quaternary gene pool of common beans.

Genetic diversity of tepary beans detected with microsatellites

The microsatellites were very useful at distinguishing tepary beans and correctly grouped the tepary beans into the species *P. parvifolius* and the variants of *P. acutifolius* (cultivar, wild var. *acutifolius* or wild var. *tenuifolius*). The designation *P. acutifolius* var. *latifolius* was again shown to be invalid as was found in Muñoz et al. (2006). The microsatellites showed low diversity within most of the cultivated *P. acutifolius* and gave a possible origin for the domesticated cultivars in Sinaloan accessions from wild *P. acutifolius* var. *acutifolius*.

One advantage of this study was that the microsatellites used detected much greater differences within *P. acutifolius* than the AFLPs used by Muñoz et al. (2006), probably because they represented inherently more polymorphic loci than the AFLP bands. A larger number of genotypes were evaluated with the microsatellite markers and overall this was an easier marker system to work with than the AFLPs.

However, as with AFLP data, the microsatellite information predicted that *P. lunatus*, followed by *P. vulgaris* were the most distant groups from *P. acutifolius* (data not shown from UPGMA analysis). Within both *P. vulgaris* and *P. lunatus* the distinction between Andean and Mesoamerican gene pools was clear as described above, and these groups shared only 35 % similarity in the microsatellite analysis.

Overall the structure of the *P. acutifolius* dendrogram made with microsatellites agreed with that of the AFLP results of Muñoz et al. (2006) and correlation between the matrices generated for AFLP and microsatellite datasets was high ($r = 0.703$) as indicated by the approximate Mantel test ($t = 12.15$, $P = 0.0198$). Notably for microsatellites, all the cultivated *P. acutifolius* formed a cohesive group with the highest genetic similarity of around 85 % indicating close relationships. One group of cultivars from Sinaloa had 100 % similarity showing no distinction at the molecular level.

Relationships among tepary beans

The relationships within the *P. acutifolius*–*parvifolius* clade have been controversial. The AFLP data from Muñoz et al. (2006) suggested that the *P. acutifolius* and *P. parvifolius* probably do not deserve to be different species, but could qualify as possible subspecies or variants within the species. The microsatellite data meanwhile showed that wild *P. acutifolius* accessions and the *P. parvifolius* accessions are the extremes of a continuum, with all of the *P. a. var. tenuifolius* accessions as intermediates between these two clusters.

The population structure analysis provides validity for the separateness of the species *P. parvifolius* since there was no evidence of admixture with any of the variants of *P. acutifolius*. The similarity of the variants *P. acutifolius* var. *acutifolius* and var. *tenuifolius* was observed by several previous authors (Baudet 1977, Maréchal et al. 1978; Buhrow 1983; Schinkel and Gepts 1989) while the distinctness of *P. parvifolius* is accepted by Freytag and Debouck (2003).

The microsatellite information was also useful in showing that only one domestication event is likely to have occurred for tepary bean. This contrasts with the results of Muñoz et al. (2006) where all wild and cultivated tepary beans formed a cluster and the domestication site was not easy to distinguish. In that study, the authors were not able to determine the likely origin of the cultivars from the wild tepary beans due to limited polymorphism within the cultivars but did find that the cultivated types seem to be associated with the Sinaloa or Jalisco regions of Mexico. In this study we were able to narrow down the probable source to the Sonoran desert region of Sinaloa since the

wild tepary accessions with most similarity to cultivated types were from this region and were of the variety *P. acutifolius* var. *acutifolius*.

The microsatellites show in contrast to the AFLPs that there has been very little introgression from wild relatives into the cultivated gene pool after the initial domestication event. This was to be expected since tepary beans are known to have a very low out-crossing rate that limits the creation of new diversity within the crop (Garvin and Weeden 1994). The lack of diversity within the cultivated tepary bean is a serious limitation for improvement of the crop and contrasts with some of the variability found for disease and insect resistance within the species (Coyne et al. 1963, Miklas and Santiago 1996; Miklas et al. 1998; Urrea et al. submitted). These are also fast evolving characteristics so could be expected to have been generated by mutation even without a lot of initial diversity or inter-crossing.

Other authors have also shown that the genetic diversity within the cultivars of tepary bean is quite low; in studies of phaseolin pattern (Schinkel and Gepts 1988) or isozyme (Schinkel and Gepts 1989; Garvin and Weeden 1994) and AFLP markers (Muñoz et al. 2006) diversity is limited. Somewhat greater diversity is found in wild relatives both within the foliar variants of the species (*P. acutifolius* var. *acutifolius*, *P. acutifolius* var. *latifolius* and *P. acutifolius* var. *tenuifolius*) and the wild relative species *P. parvifolius*. This suggests that a bottleneck occurred when the species was first domesticated and that only a subset of the wild germplasm was involved in selection.

Further, evidence of a domestication bottleneck is the very limited range of seed colors, shapes, sizes and patterns found in tepary beans with small white seed being predominant and a few accessions having yellow or stippled seed (Freytag and Debouck 2002). Meanwhile, that lack of diversity in other characteristics of cultivated tepary beans such as plant morphology and adaptation range has serious implications for improving the species and suggests that the use of wide crosses, inter-specific and inter-varietal hybridization would be very useful to improve tepary beans into a modern crop. Indeed, the high diversity found in the wild accessions of tepary bean compared to the cultivated accession suggests that the wild *P. acutifolius* genotypes are a rich source of new alleles for tepary bean improvement. For example, wild tepary bean could be used to study the level of potyvirus or geminivirus resistance in the species since virus infection is problematic (Miklas and Santiago 1996). However, wild tepary beans are very small seeded and have gray color with a stippled pattern that is hard to use in breeding.

Use of microsatellite fingerprinting for tepary beans

The evaluation of tepary bean germplasm through an accurate fingerprinting technique such as microsatellite

evaluation is very timely as only 300 cultivated and 150 wild accessions of tepary beans are stored in the FAO germplasm bank with many of these being duplicates of holdings in Mexico and the United States (Pratt and Nabhan 1988). In this study, we analyzed about one-third of these tepary beans and therefore our study was very comprehensive. For some cases we found certain accessions within the FAO collection to be duplicate genotypes. The discovery of duplicates by microsatellite alleles is more accurate as a method of genetic fingerprinting compared to morphological evaluation or AFLP analysis where polymorphism is low (Muñoz et al. 2006).

In general, microsatellites were shown to be very complementary to the AFLP analysis of Muñoz et al. (2006). The use of two marker systems to sample different parts of the genome that evolve at different rates has given us a more accurate picture of the relationships within and between the subgroups of tepary beans. The high similarity among all the cultivated tepary beans, even with the microsatellites, seems to indicate that the crop may have arisen from a single domestication event that led to a genetic bottleneck which limits diversity within the cultivars. This confirms results from AFLP analysis of Muñoz et al. (2006). Finally, the microsatellites were better at distinguishing the variants of *P. acutifolius*, namely var. *acutifolius* and var. *tenuifolius* than were the AFLP markers.

Domestication of tepary beans

Several researchers have had an interest in the wild populations that gave rise to cultivated tepary bean (Nabhan and Felger 1978; Manshardt and Waines 1983; Schinkel and Gepts 1988, 1989; Garvin and Weeden 1994). Most of these studies predict that domestication of tepary bean occurred about 5,000 years ago near the United States-Mexico border with the crop spreading north and south as recently as 1,200 years ago. This would be in agreement with our finding that cultivated tepary bean most resembles wild tepary beans from the Sinaloa-Sonoran desert region of Mexico. Wild accessions of tepary bean are commonly found in various departments of northern Mexico but fewer are found in the United States (Freytag and Debouck 2002). Finally, archeological remains of early domesticates are found in Hohokam, New Mexico and Tehuacán in Central Mexico showing the geographical extremes to which tepary bean reached at a minimum in the era before present (Kaplan 1967; Kaplan and Lynch 1999).

Present day cultivation and even oral history for tepary bean cultivation is limited as in modern times the crop has largely been abandoned, although some remnant cultivation remains. For example, among the Pueblo tribes of North America, tepary bean sometimes substituted

common bean in the “three sisters” (pumpkin, maize and beans) and tepary beans are still grown in some areas. The crop was traditionally grown by Amerindian peoples including the Maya, Maya Itza, Opata, Papago, Pueblo, Seri, Tarahumar, Tephuan, Quinche and Xinca tribes and civilizations (Debouck and Smartt 1995).

The disappearance of most of these pre-hispanic cultures has led to the loss of tepary bean diversity. In addition, it does not appear that European descendants added much to cultivated tepary bean, nor has a modern breeding program ever been established for the crop. The high amounts of diversity found in the wild *P. acutifolius* and *P. parvifolius* accessions are interesting resources for breeding tepary bean cultivars which seem to be low in diversity.

Therefore, one practical conclusion of this work is that cultivars of tepary bean could be crossed with wild accessions and with *P. parvifolius* genotypes to introgress more diversity and perhaps capture new alleles for variability in seed size, seed color, plant morphology, etc. A useful method for introgressing these novel alleles would be advanced backcross-quantitative trait locus analysis as was conducted for wild and cultivated common bean by Blair et al. (2006).

The understanding of diversity can lead to improved use of tepary bean in crosses within the species or across interspecific boundaries. The lack of diversity within the cultivated accessions suggests that more emphasis should be placed on collecting and preserving the wild relatives that are left. The results of the microsatellite analysis described here probably apply to the crop in general since about half of both the wild and cultivated collections of tepary beans held by FAO were evaluated in this study and remaining diversity outside this collection is low.

Another main finding was that the microsatellites could distinguish between *P. acutifolius* var. *acutifolius* from *P. acutifolius* var. *tenuifolius*, but these two forms can visually be distinguished morphologically so markers would not be needed to confirm intra-specific crosses. On the other hand, markers would be quite useful in *P. acutifolius* var. *tenuifolius* by *P. parvifolius* crosses where hybrids are hard to identify.

In summary, we found that cultivated tepary bean is not very diverse especially compared to its immediate wild relatives probably due to a single domestication event and genetic bottleneck. Wild tepary beans, *P. acutifolius* var. *acutifolius* and var. *tenuifolius*, on the other hand had wide diversity as did the accessions of *P. parvifolius* that were tested. *P. parvifolius* was found to be most likely a separate species rather than a variant of *P. acutifolius* agreeing with results of Florez et al. (2003). The variants for wild *P. acutifolius* of var. *acutifolius* and var. *tenuifolius* also separated well in the first germplasm group suggesting validity of these sub-populations with different leaf

morphologies in agreement with Delgado (1985) and with Pratt and Nabhan (1988).

Finally, the establishment of genetic markers and diversity studies in tepary beans is especially important since both cultivated and wild tepary beans contain many favorable characteristics such as high heat and drought tolerance. Although a minor crop, tepary beans deserve to be subjected to a plant improvement program given their advantages of better genetic transformability and innate drought, heat and salt tolerance.

Acknowledgments We thank Lucy M. Díaz for technical help with software analysis, Daniel Debouck of the bean gene bank for his helpful advice on the accessions analyzed in this study; Orlando Toro and the group at the Genetic Resources Unit for providing seed; and Agobardo Hoyos, Alcides Hincapie and Guillermo Ortíz for growing genotypes for DNA extraction. The writing of this paper was supported by the IAEA grant on tepary bean improvement and by the National University of Colombia.

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