Theor Appl Genet (2005) 110: 1275–1283
DOI 10.1007/s00122-005-1964-1

ORIGINAL PAPER

Nicola Tosti · Valeria Negri

On-going on-farm microevolutionary processes in neighbouring cowpea landraces revealed by molecular markers

Received: 27 July 2004 / Accepted: 14 February 2005 / Published online: 1 April 2005

Abstract A knowledge of existing levels of diversity is fundamental for planning in situ (on-farm) conservation activities. Three neighbouring cowpea landraces (LRs) currently cultivated in central Italy were studied by amplified fragment length polymorphism (AFLP) and selectively amplified microsatellite polymorphic locus (SAMPL) markers to determine the distribution of genetic variation within and among them. The three LRs studied, although relatively similar, are highly different from one another as shown by the significance of the Fisher exact test for the genic differentiation and the absence of genotype sharing among them. Data obtained from the AFLP and SAMPL markers separately and their combined data revealed a relatively high level of diversity still present within the LRs. The more efficient SAMPL technique was better at discriminating between the plants than the AFLP markers. The three LRs studied appear to be structured as a metapopulation in which a substantial differentiation is maintained at the subpopulation level. A complex interaction of factors (drift, LR isolation, farmer selection, migration within LRs) explains the observed pattern of diversity. The results suggest that the best strategy for maintaining diversity in the area is to preserve each of the LRs observed on the farms from which it came.

Communicated by H.C. Becker

Present address: N. Tosti
& Lab srl, via Strozzacapponi 89a,
Castel Del Piano, 06071 Perugia, Italy

V. Negri (✉)
Dipartimento di Biologia Vegetale
e Biotecnologie Agroambientali (DBVBA),
Universitá degli Studi, Borgo XX Giugno, 74,
06100 Perugia, Italy
E-mail: vnegri@unipg.it
Tel.: +39-7-55856218
Fax: +39-7-55856224

Introduction

Landraces (LRs), which are of great interest for use in future breeding programs and for developing new farming systems as well as reflecting an aspect of the cultural identity of a particular group of people, should be preserved for future generations. During the last few decades, the germplasm of crop LRs and of their wild relatives has been conserved by ex situ methods (i.e. the conservation of biological diversity outside of its natural habitat). More recently, in situ (on-farm) conservation (the conservation of biological diversity in its natural habitat) has been proposed as being the best conservation strategy because it allows evolutionary processes to continue. In contrast, these processes are halted under ex situ conservation.

A knowledge of the existing levels of among-LR and within-LR diversity is fundamental for planning in situ conservation activities. If genetically similar LRs exist in a certain area, the conservation activity could be carried out on a single farm. If, however, the LRs are different, several farms would be needed to effect their preservation. The level of variation within a population is also important because it affects the persistence of the population over time (Nunney and Campbell 1993; Soulé 1987). Changes in diversity can be monitored by assessing variation, the results of which would verify whether the conservation measures adopted are effective.

On-farm conserved diversity is subject to a wide range of forces (human, biological, edaphic and climatic), but available data (both descriptive and hypothesis testing) are still too limited to formulate a rationale for optimal on-farm management (Brown 2000). This is especially true for on-farm conservation of LRs that have been introduced and maintained outside of the domestication area.

Cowpea [*Vigna unguiculata* subsp. *unguiculata* (L.) Walp.] is an important grain legume that is cultivated throughout all of the tropical and subtropical regions as well as in the southern parts of Europe and the United

States. Cowpea was domesticated in the sub-Saharan area around the second millennium B.C. and was later introduced into Europe. It was cultivated by the Greeks in the third century B.C. and by the Romans in the first century A.D., at which time it was called "*phaseolus*". The introduction of *Phaseolus vulgaris* L. as a food crop and the recent changes in the manner food is produced, exchanged and consumed have resulted in a strong reduction in cowpea cultivation in Europe, with a consequent loss of genetic diversity. In Italy, cowpea is currently considered to be a minor crop; its cultivation is based on LRs and is restricted to a very limited acreage.

The LRs studied in this investigation have a particular cultural identity that is tied to the human populations living around Lake Trasimeno in Umbria (central Italy) where this species has been grown for centuries. The cowpea LRs in this area differ from each other with respect to morpho-agronomic, physiological and genetic traits and are generally cultivated in small fields (about 500 plants) by different farmers. Seed exchange between farmers is infrequent, and when it does occur it is usually because a farmer has accidentally lost his own particular seed (Negri et al. 2000; Negri and Tosti 1997).

The distribution of genetic variation both within-populations and between-populations is determined by mutation rate, gene flow, selection, mating system and genetic drift. Of these, only mutation and gene flow from different populations can increase the allelic diversity in a certain population. In Italy, the gene flow between local cowpea LRs can only occur with other cultivated populations since wild progenitors and modern cultivars are not present. The mating system of the species, known as a prevalent selfer (Kumar et al. 1976; Williams and Chambliss 1980), suggests that gene exchange is a sporadic event, but this should be assessed in each area of cultivation since cross-fertilization could be relatively high (Westphal 1974), especially in humid areas (Purseglove 1968).

The isolation of the cowpea LRs of this area (no gene flow from wild progenitors or cultivated varieties, rare seed exchange among farmers, clear separation of the fields) and the restricted area studied provide a good model for studying the effect of prolonged in situ management on genetic diversity. Moreover, although many studies exist on the population structure of wild species, information on nearby populations of cultivated selfers is sparse. The aim of this study was to obtain information that can be used to design an optimal on-farm conservation strategy.

The amplified fragment length polymorphism (AFLP) and selectively amplified microsatellite polymorphic locus (SAMPL) techniques were used to obtain this information. The AFLP technique has already been used to evaluate genetic diversity in wild and cultivated accessions of *V. unguiculata* (Coulibaly et al. 2002) and to construct maps (Menendez et al. 1997; Ouedraogo et al. 2002). The SAMPL technique (Morgante and Vogel 1994) extends the capabilities of the AFLP marker system because it also amplifies microsatellite (simple sequence repeat) loci (using one AFLP primer in combination with a primer that is complementary to the sequence of two adjacent SSRs) without prior cloning and characterizing of the specific microsatellite sequences. SAMPLs are often more polymorphic than other types of molecular markers and can be useful for intraspecific comparisons (Witsemboer et al. 1997). Both techniques are able to detect even a single base mutation, have a high multiplex ratio and have been efficiently used to detect diversity among cowpea LRs from the Trasimeno Lake area (Tosti and Negri 2002).

Materials and methods

Plant material and DNA isolation

The seeds from three LRs (indicated hereafter as BOS, FRE and MAR based on the initials of the respective farmer) were collected from three different farmers around Lake Trasimeno (Umbria region, central Italy) within a relatively restricted area (Fig. 1). Details on the physical and climate characteristics of the collection

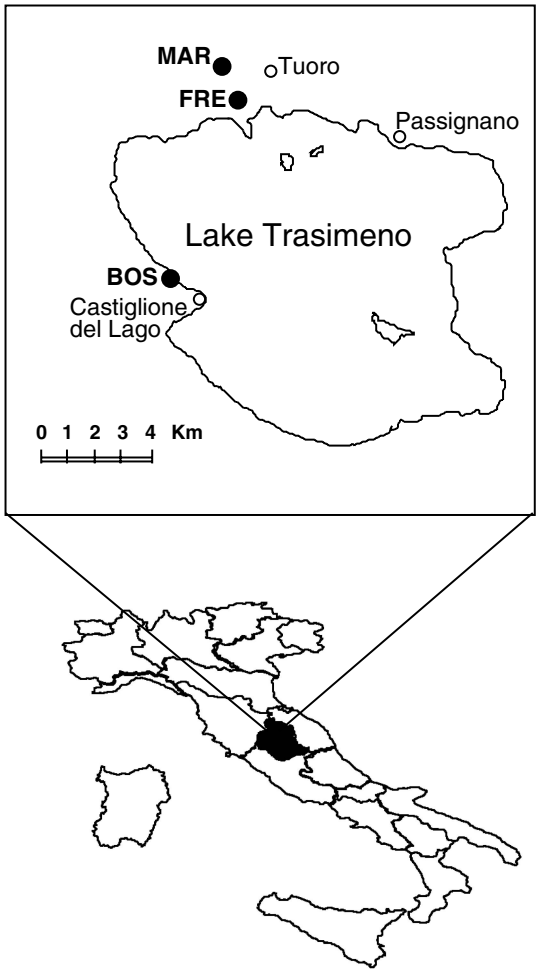

Fig. 1 Collection sites of the LRs studied

sites can be found in Negri and Tosti (1997). One hundred seeds per LR were germinated in petri dishes, and the seedlings were subsequently transplanted into jiffy pots and grown in the greenhouse. The DNA was isolated from 90 individual plants (30 for each LR) following the protocol of Doyle and Doyle (1990). The quality of the DNA was checked by electrophoresis on a 1% agarose gel containing ethidium bromide (0.5 μg/ml) in 1× TBE buffer (89 m *M* Tris-HCl, 89 m *M* boric acid, plus 2 m *M* EDTA). The DNA was quantified using a DU650 Spectrophotometer (Beckman Coulter, Fullerton, Calif.).

AFLP assay

The AFLPs were generated according to the protocol of Keygene (Vos et al. 1995). Genomic DNA (0.5 μg) was digested with 5 U *Eco*RI and 5 U *Mse*I (New England BioLabs, Beverly, Mass.). For the ligation of the adapters, 5 pmol of *Eco*RI adapter, 50 pmol of *Mse*I adapter and 1 U of T4 DNA ligase (Pharmacia, Piscataway, N.J.) were added. The primers used (GibcoBRL, Gaithersburg, Md.) in the pre-amplification step included the respective restriction enzyme adapter sequence plus a recognition site and one-base extension (C for the *Eco*RI primer and A for the *Mse*I primer). The product was used as the template for selective amplification, using primers with two-base extensions. Five primer combinations were used (Table 1), and in each case, the *Eco*RI primers were fluoresceine-labelled. The thermal cycle conditions applied in both amplification phases were the same as those used by Vos et al. (1995). An 8-μl aliquot of modified formamide dye (98% formamide and 10 m *M* EDTA with bromophenol blue) was added to the total reaction. A 6-μl aliquot of each reaction product was then heated for 5 min at 95°C and subsequently analysed on 6% denaturing polyacrylamide (19:1) gels with 7 *M* urea in 1× TBE buffer. Electrophoresis was performed at a constant power of 95 W for 2 h and 15 min on a Genomix LR apparatus (Beckman). The same equipment was used to obtain the fluorescent images of the gel following the run.

Table 1 AFLP and SAMPL primer combinations used

Name	Sequence
AFLP primer	
*Eco*RI (E)	5′-CTCGTAGACTGCGTACCAATTC
*Mse*I (M)	5′-GACGATGAGTCCTGAGTAA
SAMPL primer	
AS1	5′-$(CA)_7$CTATAT
AS2	5′-$(GA)_8$TA
AFLP and SAMPL primer combinations and selective bases	
E + CAA/M + ACT	AS1/M + AGA
E + CAA/M + ACC	AS1/M + AAT
E + CAA/M + AGC	AS1/M + AAA
E + CAC/M + ACA	AS2/M + ATA
E + CAC/M + ATA	AS2/M + ACA

SAMPL assay

The reaction products derived from the AFLP pre-amplification were diluted tenfold, and 5-μl aliquots of this dilution were amplified using combinations of a SAMPL primer and an AFLP-*Mse*I primer. The thermal cycle conditions were the same as those described for the AFLP protocol except that the annealing temperature of 56°C for the first cycle, was reduced 0.7°C for each of the subsequent 11 cycles and then stabilized at 49°C for the remaining 19 cycles. Two different SAMPL fluoresceine-labelled primers (Genset) in combination with five different *Mse*I primers were used (see Table 1). The SAMPL primer sequences were those described by De Simone et al. (1997).

For both markers, the samples were amplified and run following a randomization scheme (i.e. three replicates of ten plants each per LR). Two separate amplifications and runs were made for all of the samples and primer combinations in order to have reliable data.

Data analysis

Alleles detected and population structure

Only distinct, amplified polymorphic bands found in both replicate experiments were considered when scoring the data; faint bands were ignored. We assumed that each band that was detected corresponded to a dominant allele at a locus. Because the species that was being studied is a selfer, we also assumed that all individuals were homozygous at that locus and, consequently, that no information was missed due to the dominance of the markers used.

For each marker, the number and percentage of polymorphic loci was calculated in each population and in all populations taken together. The number of genotypes was also counted.

The distribution of alleles among the various populations for the loci detected was then arranged in a contingency table, and the significance of the distribution across populations for the overall loci (genic differentiation) was tested by means of an unbiased estimate of the *P*-value of the probability test (Fisher exact test) using the GENEPOP software (Raymond and Rousset 1995). The same software was used to test the linkage disequilibria (LD) for each pair of loci in each population.

Among-population and within-population gene diversity for both types of markers were estimated by the Shannon Index. This technique is appropriate for dominant markers and is independent of the assumption of equilibrium and knowledge of the levels of selfing, especially when the sample size is relatively high and the number of individuals per population is equal, as in the present study (Bussel 1999). The Shannon Index was calculated as $H_j = -\sum p_I \log_2 p_I$, where p_I is the frequency of a given AFLP or SAMPL fragment. The

average diversity over all the populations was calculated for each locus as $H_{pop} = 1/n\sum(H_j)$, where n is the number of populations. Total diversity was calculated as $H_t = -\sum p_s \log_2 p_s$, where p_s is the frequency of a fragment over all the samples from the three populations. The proportion of diversity within populations was estimated as $H_{wp} = H_{pop}/H_t$, and the proportion of diversity among populations was estimated as $H_{ap} = 1-(H_{pop}/H_t)$.

In order to compare our results with data reported elsewhere, Nei's gene diversity for each population (H_S) and for the whole sample (H_T) and the $G_{ST} = 1-(H_S/H_T)$, an estimate of genetic differentiation among populations (population subdivision), were also calculated (Nei 1973). A Spearman's rank correlation between the Shannon Index and Nei's gene diversity estimates were worked out in order to evaluate the correspondence of the results obtained.

Pairwise genetic distances between LRs (Nei 1972) were also calculated. Numerical resampling was used to estimate value significance using the GENETIX 4.04 software (Belkhir et al. 1996–2002).

Comparisons among marker systems

To assess the correspondence of the results obtained with the two different marker systems, we conducted the Mantel matrix-correspondence test on the similarity matrices obtained with AFLP and SAMPL markers. For each type of marker, the genetic similarities were only calculated on the polymorphic loci using the Dice coefficient: $D = 2a/(2a + b + c)$, where a is the number of shared bands and b and c are the number of bands present in one sample but absent in the other sample. The analyses were performed using the NTSYS.PC package ver. 1.8 (Rohlf 1993).

The diversity assessed at different markers results from either "marker-specific factors" (such as mutation) or "marker-non-specific" factors (for example, drift or migration). Populations whose evolutionary history mainly involved drift and/or migration differ with respect to their level of diversity in the same way for different kinds of markers. Different results are obtained if mutation is the prime determinant of diversity. In other words, congruent between-marker rankings are expected when "marker-non-specific factors" are involved, while the opposite is true when "marker-specific factors" are involved (Mariette et al. 2001). To test which type of factor was the principle factor in determining diversity, a Spearman's rank correlation was calculated between the Shannon's and Nei's within-population diversity estimates relative to AFLP and SAMPL.

Relationships among the genotypes detected

In order to summarize the relationships among the genotypes detected with the two marker systems, we used the similarity matrices to perform a principle coordinate analysis (PCoA). A minimum spanning tree (MST) was then superimposed. Both analyses were worked out using the NTSYS.PC package ver. 1.8 (Rohlf 1993).

Finally, to support the connections between genotypes that resulted from MST with statistical evidence, we assigned individuals to each LR by using the likelihood-based Bayesian technique implemented in GENECLASS (http://www.montpellier.inra.fr/CBGP/softwares). In particular, to assess the probability of a multilocus genotype being derived from the allele frequencies of a certain LR, the likelihood of each individual genotype was compared to the distribution of the likelihood of 10,000 randomly generated genotypes.

Results

Alleles detected and population structure

Irrespective of the technique used, a similar number of bands (loci) was detected with the AFLP and SAMPL markers (197 and 192, respectively) (Table 2), but SAMPL revealed a higher proportion of polymorphic bands (17.71% vs. 7.11%) and more genotypes (84 vs. 34 from 90 samples) than AFLP. However, both techniques revealed a similar distribution of the polymorphic fragments within the LRs, with BOS and FRE showing a higher proportion of polymorphic bands than MAR. In contrast, the number of genotypes within each LR was different depending on the marker system applied. In particular, of all three populations, FRE showed the largest number of discriminated genotypes with AFLP (17) and the least with SAMPL (26), while MAR had the fewest identified genotypes (5) with AFLP and the highest (29) with SAMPL (Table 2). It is worth noting that regardless of the marker techniques used, no shared genotypes were found among populations.

With the partial exception of MAR (AFLP data), polymorphism within populations was quite high, i.e. over 50% of the loci were not fixed. Most of the allele frequencies were very different among the LRs (data not shown) and the Fisher exact test taken over all of the loci examined was highly significant ($P = 0.0001$), which indicates a strong differentiation among the LRs.

The population genetic diversity estimated by the Shannon Index and Nei's gene diversity and the partitioning of variation are presented in Table 3. Both indices yielded similar results (Spearman's rank correlation of 0.943, $P = 0.0350$). A strong among-population differentiation and a consistent level of within-population diversity were found with both markers, but a higher variation within LR was detected with SAMPL than with AFLP.

The within-population genetic diversity of MAR and FRE was also assessed differently depending on the marker technique used: MAR had the least diversity using AFLP markers and FRE had the least diversity with the SAMPL markers. In contrast, BOS was the most variable population with both marker techniques.

Table 2 Banding and genotype patterns generated by AFLP and SAMPL marker systems in the whole experiment and in single LRs

	AFLP			SAMPL		
	Total (*n*)	Percentage overall	Percentage within LR	Total (*n*)	Percentage overall	Percentage within LR
Banding pattern[a]						
Bands detected overall	197			192		
Polymorphic bands overall	14	7.11		34	17.71	
FRE	8	4.06	57.14	24	12.50	70.59
BOS	10	5.08	71.43	24	12.50	70.59
MAR	2	1.02	14.29	19	9.90	55.88
Genotype pattern[b]						
Individuals studied in total	90			90		
Individuals studied in each LR	30			30		
Genotypes detected overall	34	37.77		84	93.33	
FRE	17		56.66	26		86.67
BOS	12		40.00	29		96.67
MAR	5		16.67	29		96.67

[a]Total number of bands, overall and within LR proportions of polymorphic bands

[b] Total number of genotypes, overall and within LR proportions of genotypes detected

Table 3 Partitioning of genetic diversity (Shannon Index and Nei's gene diversity) generated by AFLP and SAMPL into within-components and between-components

Source of variation	AFLP	SAMPL
Shannon index		
FRE	0.178	0.188
BOS	0.271	0.265
MAR	0.051	0.216
All	0.351	0.329
Between landraces	0.525	0.323
Within landraces	0.475	0.677
Nel's gene diversity		
H_s FRE	0.157	0.138
H_s BOS	0.245	0.225
H_s MAR	0.059	0.175
H_t	0.347	0.287
G_{st}	0.557	0.375
Within landraces	0.443	0.625

Table 4 Nei's original measure of genetic distances between the landraces examined on the basis of AFLP data (above the diagonal) and SAMPL (below the diagonal) data. Significance level was $P = 0.0001$

	FRE	BOS	MAR
FRE	–	0.4020	0.3559
BOS	0.1068	–	0.4986
MAR	0.3216	0.2347	–

The Nei's pairwise genetic distance values were higher for AFLP than for SAMPL (Table 4). Differences in the level of genetic distances detected by the different markers were evident; MAR and FRE were the most distant populations with SAMPL, while MAR and BOS were the most distant populations with AFLP.

The AFLP system revealed a noticeably different pattern of LD within populations (Table 5). Excluding MAR, where only one LD value could be calculated, the most polymorphic population (BOS) had the highest proportion of significant pairwise LD (60.0%). In contrast to AFLP, SAMPL revealed low and similar LD values in all the populations (5.4%, 6.2% and 6.4% in FRE, BOS and MAR, respectively).

Comparisons among marker systems

The genetic similarity (GS) estimates obviously mirrored the genetic diversity estimates. The average GS was slightly higher for AFLP (0.7087) than for SAMPL (0.6962), the lowest average GS among individuals within LR was observed in BOS with both techniques (0.8108 and 0.7492, for AFLP and SAMPL, respectively), the highest GS value for AFLP (0.9479) was observed in MAR and the highest value for SAMPL (0.8490) was observed in FRE.

The Mantel matrix correlation calculated on the similarity matrices obtained with AFLP and SAMPL was low but significant (0.4050, $P > 0.0002$). Moreover, the Spearman's rank correlation calculated between the Shannon's and Nei's within-population genetic diversity estimates of AFLP and the estimates relative to SAMPL was not significant (0.6000, $P = 0.1797$).

Relationships among genotypes detected

If we consider the relationship $P = 1-(1-f)^n$, where P is the probability of detecting a genotype in a sample of n plants when that genotype occurs with a frequency f (Van Treuren and Van Hintum 2001), non-frequent genotypes were also detected with a 95% probability (i.e. those occurring with at least a 9.5% and 3.3% frequency in samples of 30 and 90 plants, respectively).

The results of principal co-ordinate analysis (PCoA) for AFLP, SAMPL and combined data with the superimposed minimum spanning tree are shown in Fig. 2. Dark gray, pale gray and white points refer to FRE, MAR and BOS LRs, respectively. Each point indicates one genotype except when indicated with a number. The

Table 5 Number and proportions (in brackets) of significant pairwise linkage disequilibria found in each population. Significance level was $P = 0.05$

Population	AFLP		SAMPL	
	Number of comparisons		Number of comparisons	
	Total	Significant	Total	Significant
FRE	28	1 (3.6%)	276	15 (5.4%)
BOS	45	27 (60.0%)	276	17 (6.4%)
MAR	1	1 (100.0%)	171	11 (6.4%)

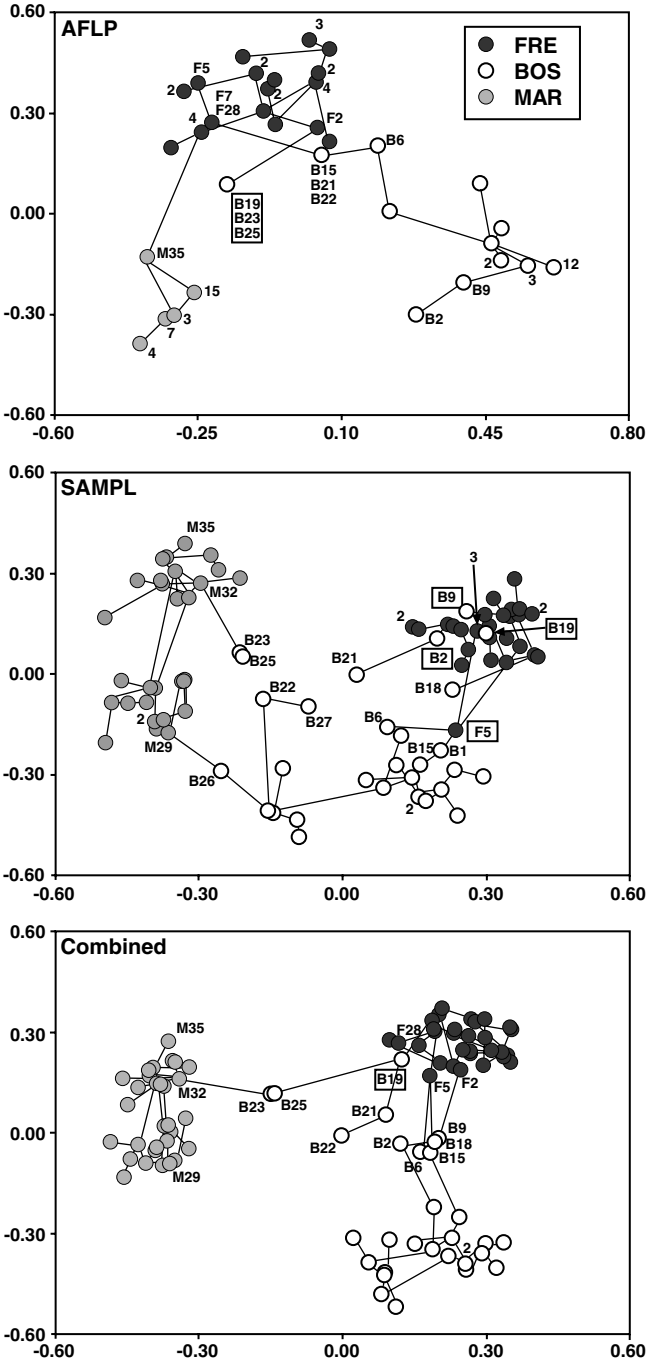

Fig. 2 PCoA for AFLP, SAMPL and combined data with the MST. *Each point* indicates one genotype except when indicated with a *number*. See text for explanation of codes

most interesting plants are indicated with their names close to the coloured point (inside the boxes those diversely allocated by assignment test); when present, the numbers indicate the number of plants assigned to genotypes.

In the PCoA, the first two axes accounted for 69.70% of the total variation in the case of AFLP (41.14% for the first and 28.56% for the second axis) and 46.89% for SAMPL (30.10% for the first and 16.79% for the second axis).

In the case of AFLP, FRE and MAR were well discriminated, while the plants from BOS appeared to be more diffuse. Two BOS genotypes (each genotype consisting of a group of three plants—B19, B23, B25, and B22, B21, B15) were more closely connected to FRE individuals than to other BOS genotypes. However, the assignment test only allocated one of these genotypes to FRE (that which corresponded to plants B19, B23 and B25).

The FRE and MAR populations were also better discriminated than BOS with SAMPL. The MAR plants were subdivided into two groups, and both were clearly separated from the plants belonging to the other LRs. Some BOS genotypes were spread among the two other LRs and some were allocated in a different way than with AFLP. In particular, B19, B23, and B25 were detected as different genotypes, but only B19 was assigned to FRE. Similarly, B2 and B9 (assigned to BOS with AFLP) were assigned instead to FRE, while F5 was assigned to BOS.

When combined data were used to construct principal co-ordinate diagrams, the first two axes accounted for 50.30% of the total variation (29.41% and 20.89% for the first and second axis), and a genotype grouping that was more consistent with the origins was obtained. Only three BOS genotypes (B23, B25, and B19) were spread among the other LRs. However, the assignment test allocated only plant B19 to a different origin (FRE).

Discussion

Although the overall level of similarity was quite high, there was a clear distinction among the neighbouring populations studied. In addition, despite the fact that *V. unguiculata* is a prevalent selfer, a relatively high within-population diversity was found. Selfing annual species, such as *V. unguiculata*, are thought to maintain more genetic among-population variation than within-

population variation, which is in contrast with the situation of outcrossing and/or perennial plants. Bussel (1999) reviewed the G_{st} estimates obtained with RAPDs and found an average value of 0.596 for the inbreeding species and 0.155 for the outbreeding species. These values are in agreement with those revealed by allozyme analysis for outbreeding species ($G_{st} < 0.190$), for inbreeding dicots (0.587) and for inbreeding monocots (0.412) (Hamrick and Godt 1996).

The G_{st} value found in our materials with SAMPL (0.375) revealed a higher within-population diversity than those reported in the above-mentioned studies. However, a relatively high proportion of within-population diversity has been found in other prevalently selfing crop species such as durum wheat ($G_{st} = 0.15$; Tsegaye et al. 1996), barley ($G_{st} = 0.10$; Papa et al. 1998) and flax ($G_{st} = 0.07$; Mansby et al. 2000) with RAPD and isozymes. Such a level of diversity was also found in the wild inbred *Medicago truncatula* using RAPDs and microsatellites ($F_{st} = 0.32$ and 0.37, respectively), (Bonnin et al. 1996, 2001).

Overall, our data show that cowpea from the Trasimeno Lake area appears to be structured as a metapopulation where substantial differentiation is maintained at the subpopulation level. Results from other studies in which LRs from the Trasimeno Lake area were compared with populations from abroad (Tosti and Negri 2002 and unpublished data) and the high overall level of similarity found in this study suggest that the LRs under study evolved from a single introduction gene pool.

Different sub-populations may evolve from an initial gene pool due to the effects of mutation, genetic drift, seed and gene flow and selection. Mutation creates new alleles upon which the other evolutionary forces mentioned will subsequently operate. The low correlation between the similarity matrices generated by AFLP and SAMPL suggests that the different markers detected different levels of mutation at different loci. The absence of any correlation between the diversity indices calculated on AFLP and SAMPL data indicates that the diversity observed in this study is predominantly due to marker-specific factors, such as mutation. On the other hand, the low number of polymorphic AFLP markers may have affected the comparison between marker systems, and it is difficult to comprehend why the mutation rate should be different in such a restricted area. Other evolutionary factors (drift, migration, selection and population dimension over time) should have played a role in differentiating the populations. These factors will now be considered with respect to among-population and within-population diversity.

Among the evolutionary forces, random fixation of alleles (drift) certainly operates and enhances among-population diversity, especially when coupled with selfing and a medium-to-small population size, such as those studied here. The data relative to the level of polymorphism and LD obtained with the AFLP markers show that the populations studied here have experienced different levels of fixation (compare MAR with the other populations, Tables 2, 5). On the other hand, both markers, but SAMPL in particular, show that a relatively high proportion of the examined loci are not fixed and that there must be forces which work against fixation.

In addition to the effect of drift, selective pressures could also have played a role in differentiating the populations. Selection has strongly influenced the evolution of neutral loci of a wheat composite multiplied for a few years in different environments (Goldringer et al. 2001). In addition, with population subdivisions, local selection enhances between-deme diversity even at neutral loci that are distant from polymorphic loci (Charlesworth et al. 1997).

The physical and climatic pressures are similar in the restricted area that we studied here (although microenvironmental differences probably exist), but when a crop is considered, human choices or preferences should also be taken into account. An intense and differential farmer-selection pressure exists in the area, as noted when material was collected on the farms and the LRs were characterized for morph-agronomic traits (Negri and Tosti 1997; Negri et al. 2000). Farmer-selection is mainly addressed to morphological characteristics (such as colour and seed size), but not all farmers apply the same criteria. Different farmers choose different colours and seed size, and only some choose for uniformity. For example, BOS is characterized by uniform, small, white seeds without any colour around the eye, MAR by uniform, tan seeds and FRE by non-uniform, brown-to-whitish seeds; both FRE and MAR seeds are heavier than BOS (Negri et al. 2000). Other farmer-selection pressures also exist, such as the level of fertilization and volume of irrigation. Therefore, it is feasible that different farmer-selection pressures applied for generations on the same, probably restricted, introduced gene pool have had a significant impact on the divergence found among populations.

As for migration, seed exchange rarely occurs between farmers (Negri and Tosti 1997), and distances of about 2 km separate the closest neighbouring LRs studied. This distance is usually sufficient to prevent insect-mediated cross-pollination, although wild pollinators (i.e. *Xilocopa* spp.) frequently visit cowpea flowers in the area. The absence of genotype sharing among the LRs found in this study confirms, from a molecular point of view, the lack of migration among populations. Nonetheless, a few genotypes belonging to the BOS LR and, in particular, B19, showed a confused relationship with the genotypes of FRE. This, at least, provides some evidence against a clear differentiation of the LRs, which should be briefly discussed. Since no recent migration events can be ascertained, it can be hypothesized that these genotypes retain variously assorted features of an original common gene-pool which was first subdivided and then shaped over time into different LRs by the effects of mutation, drift and selection. Moreover, the size of the initial subpopulations may have been unequal, or different populations may have experienced different reductions in size over time so that each of

them had different opportunities to evolve under the forces that came into play.

The relatively high level of within-population diversity can also be ascribed to several factors. First, a certain level of within-population migration (cross-pollination) may have occurred. This is supported by the above-mentioned observations on intense pollinator activity and by the low LD found for polymorphic alleles with the SAMPL markers. On the other hand, AFLP revealed a quite different LD between populations (Table 5), which may indicate a different level of within-population cross-pollination at the different sites where the LRs are grown. The LR showing the lowest LD (FRE) came from the hillside area where wild pollinators can find numerous refugia in semi-natural areas bordering the cultivated fields, while the LR showing the highest LD (BOS) came from the intensively used area (high human activity) nearby the lake where pollinator activity is greatly hampered.

The maintenance of different alleles within populations could also be due to the continuous changes in the selective pressures, which cause the fitness of different alleles to change with time. An allele that, at one time, was deleterious or neutral, may become favourable and selection may negate the loss of the allele due to drift (Crow 1986). It is worth noting that, more than natural selection, selection pressures applied by farmers are likely to change with time and from one generation of farmers to another. Heterozygote advantage could also have contrasted within LR fixation. Moreover, if recombinant individuals have a higher fitness, the outcrossing rate may be enhanced in the subsequent generation if it is inheritable (Bonnin et al. 2001).

The maintenance of different alleles due to a patchy spatial distribution of plants or selection acting at the micro-habitat level (which favours different genotypes in a different part of the site and gives rise to small neighbourhood sizes where it is easier to maintain overall diversity at the subpopulation level), such as suggested by Bonnin et al. (2001) for different subpopulations of *M. truncatula*, should be excluded in the case of a crop. Cowpea seeds are harvested, selected and mixed for sowing, and the field of each farmer should be considered to be a rather uniform environment.

A complex concurrence of factors appears to have determined an unexpected diversity in the restricted area that was studied. However, the extent to which the variation detected is useful for maintaining viable populations in the future has not yet been fully clarified since it is not known to what extent the molecular markers used are related to the traits under selection pressure in cultivation. These markers are assumed, but not proven, to be neutral. Molecular markers targeted to gene-controlled characters under human or environmental selection pressure should be used to the purpose. In addition, the coupling of neutral with non-neutral markers would be useful to assess the respective role of drift and selection in shaping the diversity of LRs.

Conclusion

It is a hard fact that the on-farm conservation of particular populations has to be based on an understanding of the extent of variation among and within those populations (Brown 2000). The amount of genetic variation maintained in a population may be important for the future ability of that population to adapt to new evolutionary pressures, thus affecting its long-term chances of survival.

The substantial genetic differentiation of the subpopulations (LRs) increases the effective size of the whole Trasimeno population and, consequently, its chances of survival. Since no migration occurs among LRs, any mutation arising in a LR may become fixed in that deme, but it cannot spread to other LRs. Hence, no allele can ever fix in the metapopulation; drift to fixation takes an indefinitely long time and the effective size of the metapopulation becomes infinite. When a limited migration exists, this effect is still operative, but to a lesser extent (Whitlock and Barton 1997). On the other hand, local extinction of a single LR would reduce the overall amount of variation and should therefore be prevented.

Consequently, this study suggests that the best strategy for preserving the diversity of a selfer, even in a restricted area, would be to maintain the entire metapopulation on-farm. This means that each farmer should receive appropriate advice and support to maintain his own population (i.e. each LR should be maintained on the farm from which it came). In addition, the dimension of each population should be kept as large as possible.

Acknowledgments This work was funded by the Parco Regionale del Tzasimeno and was entirely carried out at DBVBA in partial fulfilment of the PhD thesis of Nicola Tosti.

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