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Genetic diversity and population structure of six species of *Capparis* in Tunisia using AFLP markers

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ABSTRACT

In order to study the genetic diversity, the phylogeographic pattern and hybridization between six Tunisian *Capparis* species, 213 accessions of Caper were genotyped with three primer combinations of amplified fragment length polymorphism (AFLP) markers. Out of 750 fragments generated, 636 were polymorphic and 407 of them were restricted to a single species. STRUCTURE and PCoA analyses clearly separated morphologically different populations into six distinct genetic ones. The UPGMA analysis grouped the species into three main clusters: G1 grouped *C. spinosa* subsp. *spinosa* var. *spinosa* and *C. sicula* subsp. *sicula*; G2 grouped *C. ovata* subsp. *ovata* and *C. orientalis* and G3 clustered *C. zoharyi* and *C. aegyptia*. Populations from G1, G2 and G3 were mainly distributed in arid, subhumid, and semi-arid bioclimates, respectively. Additional genetic studies on *Capparis* could help to identify genes underlying speciation events and local adaptation to geographic areas leading to the development of breeding programs.

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1. Introduction

Capparis spinosa L. (the thorny caper, *Capparaceae*) is a xerophyte plant mainly exploited as a condiment to flavor foods [1–3]. It has a wide geographic distribution in the Mediterranean area and in Central Asia [4,5], and is adapted to several bioclimatic environments, from humid to Saharan [4,6–8] and to different soil types [4,8–10]. It has a high phenotypic diversity and several morphological distinct intraspecific taxa and intermediate forms were reported making its classification very ambiguous [10–13]. In addition, morphological markers

are influenced by the environmental conditions and developmental stages, which make their use limited in diversity studies. It has become obvious these days that morphological characterization of the species of *Capparis* is not sufficient to make definitive discrimination among the species, subspecies, and varieties [14]. More recently, alternate approaches, including application of appropriate molecular markers, have been increasingly adopted to address the problems in *Capparis* taxonomy. Analyses on genetic diversity and relationship among the species of *Capparis* could also provide useful information for the conservation of genetic resources and the establishment of a *Capparis* breeding program. In fact, several recent works assessed the biological activities of caper extracts (whole plant, floral roots, sheets, buds, flowers and fruits) on bacteria [15–20], fungi [19,21], nematodes [22], pests

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[23] and adventitious [24], and suggested that *C. spinosa* is a potential source of natural antioxidant molecules [3,25–27]. Genetic diversity studies could give a general guide for choosing parental lines to make suitable cross combinations for the selection of valuable traits with large possible applications in agriculture, food industry and medicine.

The inter-simple sequence repeats (ISSRs) and random amplified polymorphic DNAs (RAPDs) analysis have been the most commonly used techniques in wild and cultivated forms of *Capparis* species, revealing genetic variation among genotypes/species and/or collected sites in Iran [28], Trans-Himalayan region [29], Syria [30], Turkey [31], Egypt [32], Italy [33] and Morocco [34]. In Syria, ISSR combined with simple sequence repeat (IRAP) revealed genetic variation among 47 samples of three *Capparis* species genotypes collected from 21 locations and divided the samples into three genetic groups: *Capparis sicula*, *C. aegyptia* and *C. spinosa* [30]. In Morocco, ISSR markers identified 20 genetic groups among 90 accessions of *Capparis* spp. [34]. In Italy, Gristina et al. [33] characterized 90 wild populations and cultivated forms using ISSR markers and suggested a clear genetic distinctness between two different subspecies of *C. spinosa* at the regional level, *C. spinosa* subsp. *spinosa* and subsp. *rupestris*. However, another recent study on eight Egyptian taxa of *Capparis* and related genera (*Capparaceae*) conducted by Moubasher et al. [32] using three primers in randomly amplified polymorphic DNA (RAPD) analyses revealed four varieties of *Capparis spinosa*: *C. spinosa* var. *deserti*, *C. spinosa* var. *canescens*, *C. spinosa* var. *spinosa* and *C. spinosa* var. *inermis*. Using RAPD technique, Özbek and Kara [31] also differentiate five different varieties, *Capparis spinosa* L. var. *spinosa*, var. *aegyptia* and var. *canescens* and *C. ovata* Desf. var. *palaestina*, and var. *herbacea*, and intermediate forms between 15 Turkish natural *Capparis* populations. In the other hand, among the different molecular tools, the Amplified Fragment Length Polymorphism (AFLP) method has been extensively used for a wide range of species, including medicinal plants [35–37], to investigate the population genetic structure and assess the genetic differentiation among species [38,39]. This technique is a powerful DNA fingerprinting technology applicable to any organism without the need for prior sequence knowledge. It is a reproducible multilocus marker system with selective PCR amplification [40]. The main advantages of AFLPs are the high levels of polymorphism and high degrees of discriminative capacity for closely related accessions [41–45]. This technique was used successfully in *C. spinosa*, revealing genetic variations among 28 samples collected from six sites in the Aleppo and Lattakia provinces (Syria), and showed the presence of specific alleles for each province [46].

In Tunisia, *C. spinosa* is widely distributed. Although it has been extensively studied morphologically, *Capparis* taxonomy is also still highly controversial. Pottier-Alapetite [47] was the first to state four varieties (var. *canescens*, var. *rupestris*, var. *genuina* and, var. *aegyptiaca*) based on morphological traits. Later, Le Floc'h et al. [48], based on Innocencio et al.'s [8] and Yousfi's [49] phenotypic studies,

sustained six *Capparis* species in Tunisia: *C. spinosa* subsp. *spinosa* var. *spinosa*, *C. sicula* subsp. *sicula*, *C. orientalis*, *C. ovata* subsp. *ovata*, *C. aegyptia*, and *C. zoharyi*, a new species mentioned for the first time. More recently, Fici's [10] works, based on morphological, ecological, biogeographical characteristics and on herbarium samples, suggested another classification and grouped intraspecific taxa of *C. spinosa* into two subspecies in Tunisia based on the presence of persistent thorns: *C. spinosa* subsp. *spinosa*, the thorny morphotype, with three varieties (var. *canescens*, var. *spinosa*, and var. *aegyptia*) and *C. spinosa* subsp. *rupestris*, the inerm morphotype, with two varieties (var. *ovata* and var. *rupestris*). According to Fici [10], *C. zoharyi* is not morphologically distinct from *C. aegyptia*. Until now genetic studies on Tunisian *Capparis* were rare and limited to one variety/species or one region, not helping to resolve this unclear classification. Khouildi et al. [50] was the first to study genetically *Capparis* species in Tunisia and showed a higher variability in Tunisian caper populations compared to Italian populations using RAPD analysis. In addition, Ghorbel et al. [51] subdivided 12 Tunisian *Capparis* populations into two genetic groups belonging to inerm and thorny morphotypes on the basis of RAPDs and isoenzymatic tools.

The objective of this study were:

- to molecularly characterize a representative panel of the morphologically identified *Capparis* accessions in Tunisia by AFLP analysis;
- to study the population structure, the phylogenetic relationship between accessions, and gene flow;
- to study the correlation of the genetic diversity with geographical and ecological suitability of *Capparis* in Tunisia.

2. Material and methods

2.1. Plant materials and DNA extraction

Two hundred and thirteen caper accessions, sampled from different climate zones, were selected for this study from the collection conserved at the Tunisian National Gene Bank (see details in [supplementary material](#)). Accessions belong to six distinct caper species (*C. sicula*, *C. spinosa*, *C. ovata*, *C. orientalis*, *C. zoharyi*, and *C. aegyptia*) and were chosen based on clear morphological characterizations [48]. Sampling site and GPS coordinates were recorded for each accession.

Total genomic DNA was extracted from frozen young leaves according to a modified CTAB procedure described by Saghai-Marroof et al. [52]. DNA concentration was estimated by spectrophotometer and by electrophoresis on [1% (w/v)] agarose gel [53].

2.2. AFLP genotyping

For each accession, 10 µL of genomic DNA (500 ng) was double digested with 5U of *EcoRI* restriction enzyme at 37 °C in a final volume of 20 µL for 4 h, then with 5U of *MseI* restriction enzyme at 65 °C in a final volume of 40 µL for 4 hours. The resulting fragments were ligated to

double-stranded *EcoRI* (5 pmol) and *MseI* (50 pmol) in ligation buffer (4UT4 DNA ligase, 10XT4 DNA ligase buffer) and incubated at 37 °C for 16 h. Ligated DNA templates were further diluted (5 fold) and pre-amplification was performed using *EcoRI*+ A and *MseI*+ C primers in a total volume of 25 µL (0.20 µL of Taq, 1 µL of enzyme, 0.2 µL of dNTP, 1.5 µL of MgCl₂ and 2.5 µL of buffer). The pre-amplification reaction was carried out in a thermocycler for 2 min at 94 °C, 30 cycles at 94 °C denaturation (30 s), 56 °C annealing (30 s) and 72 °C extension (1 min) and a final hold at 72 °C for 10 min. Pre-amplified DNA was analyzed by 1% agarose gel electrophoresis. The pre-selective amplification product was diluted 25× in a TBE buffer 1X and stored at 4 °C for amplification, or stored at –20 °C for later use. Selective amplification was performed in a final volume of 20 µL containing 1.5 µL of buffer, 0.2 µL of dNTP, 1.25 µL of MgCl₂, 0.2 µL of Taq, 5 ng of *EcoRI*+ AXX and 30 ng of *MseI*+ CYY and 5 µL of a diluted pre-amplified DNA sample. Amplifications were conducted using a touchdown PCR program: 1 cycle of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s, then 13 cycles with the annealing temperature lowered by 0.7 °C per cycle, followed by 23 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s. One microliter of the amplified product was mixed with 13.5 µL of deionized formamide and 0.5 µL of GeneScan-500 Liz internal size standard, denaturated at 95 °C for 5 min and analyzed by capillary electrophoresis on an automated ABI Prism 3130 DNA sequencer.

A screening of 12 combinations of four *EcoRI* primers (E-AAC, E-ACC, E-ACA and E-AAG) and six *MseI* primers (M-CTC, M-CAG, M-CAT, M-AGG, M-GCT and M-CAA) with three selective nucleotides were performed. Three highly polymorphic primer combinations were selected: E-AAC/M-CAG, E-AAG/M-CAA and E-ACC/M-CAT (Table 1).

2.3. AFLP polymorphism analysis

Clear and unambiguous bands in length ranging from 50 to 500 pb were considered as usable. AFLP bands were scored, across all genotypes, for presence (1) or absent (0) and transformed into 0/1 binary matrix. The total number of bands was calculated for all primers. Polymorphic bands were only taken into account to estimate the percentage of polymorphic bands (%PB). The ability of the most informative primers to discriminate among cultivars was assessed by the resolving power (Rp) [54]. Evaluation of the Rp was performed according to the formula of Gilbert et al. [55]. In

addition, the discriminating power of derived markers was made by the assessment of the polymorphism information content (PIC) using Lynch and Walsh [56] formula.

2.4. Population structure analysis

Population genetic structure was assessed using the Bayesian clustering algorithm implemented in STRUCTURE version 2.3 [57,58]. The program infers the number of populations into which the analyzed genotypes can be divided. The samples were analyzed assuming the admixture ancestry model with *K* from 1 to 10 applying 4 independent runs for each of the different values of *K*. A burn-in period of 50,000 and Monte Carlo Markov Chain replicates of 100,000 iterations were performed. The run with maximum likelihood was used to assign individual genotypes into groups. Within a group, genotypes with affiliation probabilities (inferred ancestry) ≥ 80% were assigned to a distinct group and those with < 80% were treated as “admixture”. Plot of mean posterior probability (ln *P*(*D*)) values per clusters (*K*), generated by the STRUCTURE program [57] and delta-*K* method of ln *P*(*D*) [59] were used to determine the optimal number of clusters. Principal coordinate analysis (PCoA) was also performed via distance matrix to describe the relationship between accessions using software GenAlEx version 6.5 [60].

2.5. Genetic diversity, genetic distances and phylogeny

The significance of population differentiation clustered by STRUCTURE was further investigated by performing an analysis of molecular variance (AMOVA). The gene flow was estimated by using the equation $Nm = \frac{(1-F_{st})}{4F_{st}}$, where *Nm* is the number of migrants per generation, *N* is the effective size of a population and *m* is the rate of migration. The analysis of molecular variance (AMOVA) and the determination of pairwise population genetic differentiation (*F*_{st}) and gene flow (*Nm*) were calculated by GenAlEx 6.502 [60]. Diversity indices within each subpopulations of caper were also assessed by calculating the number of segregating AFLP bands (*S*), the epiallele frequencies (*p*_{*s*}), Watterson's theta (*θ*), pi (π), the number of specific alleles in each species and the average divergence over AFLP fingerprint pairs in same subpopulation (*d*), using the software MEGA version 6 [61]. Pairwise Nei's genetic distances between *Capparis* populations were calculated using MEGA version 6 [61]. The similarity matrix was used to construct a dendrogram by the Unweighted Pair Group

Table 1

Number of AFLP bands, percentage of polymorphic bands, polymorphism information content (PIC) and resolving power (Rp) for each AFLP primer combination used on 213 *Capparis* accessions from Tunisia.

| Primer pairs | Total AFLP bands (No.) | Polymorphic AFLP bands (No.) | Percent Polymorphism (%) | PIC | Rp | Fragment size range (bp) |
|--------------|------------------------|------------------------------|--------------------------|-------|--------|--------------------------|
| E-AAC/M-CAG | 262 | 203 | 77.48 | 0.242 | 67.06 | 50–477 |
| E-AAG/M-CAA | 233 | 210 | 90.12 | 0.254 | 74.25 | 53–480 |
| E-ACC/M-CAT | 255 | 223 | 87.45 | 0.262 | 81.9 | 57–497 |
| Total | 750 | 636 | – | – | 223.21 | – |
| Average | 250 | 212 | 85.02 | 0.252 | 74.40 | – |

AFLP: amplified fragment length polymorphism.

Method Arithmetic (UPGMA) procedure using the software MEGA version 6 [61]. In addition, correlations between the genetic and the log (1 + geographic distance) transformed geographic distances of accessions were analyzed using a Mantel test for the entire population as well as for each genetic cluster individually [62]. In order to investigate the genetic variability explained by the bioclimate, an analysis of molecular variance (AMOVA) was performed under GenAlEx 6.502 [60]. The number of hybrids (*hy*), the number of segregating AFLP bands (*S*), and the average divergence over AFLP fingerprint pairs in same subpopulation (*d*) were calculated within each bioclimatic stage using the software MEGA version 6 [61]. The correlations between these diversity indices were assessed by calculating the Pearson correlation coefficient *r*.

2.6. Neutrality test

The Tajima's *D* test is a widely used test of neutrality in population genetics to assess the random or non-random evolutionary process in a species [63]. Tajima's *D* [63] test was performed for each population individually using Mega 6 software. Tajima's statistic computes a standardized measure of the total number of segregating sites in the sampled population and the average number of polymorphic sites between pairs in a sample. The null hypothesis of the Tajima's *D* test is neutral evolution in an equilibrium population. The theoretical distribution of Tajima's *D* (95% confidence interval between -2 and +2) assumes that polymorphism ascertainment is independent of allele frequency. Positive values of Tajima's *D* suggest an excess of common alleles, which can be consistent with balancing selection, population contraction or bottleneck; whereas negative results are indicative of a population that has undergone a recent expansion, as rare alleles are more common than expected.

3. Results

3.1. AFLP polymorphism

Three primer combinations generated a total of 750 reproducible amplification fragments across all caper

accessions among which 636 were polymorphic. The average number of polymorphic bands scored per primer pair was 210 (Table 1). All the tested primers were powerful to detect DNA polymorphisms in *Capparis* genus with a similar level of polymorphism. The largest number of polymorphic bands 223 was produced with primer combination E-ACC/M-CAT and the least number of polymorphic bands 203 was detected using primer combination E-AAC/M-CAG. Moreover, estimates of the resolving power (*R_p*) showed a high rate of collective *R_p* (223.21) with an average of 74.4. The Polymorphism Information Content (PIC) ranged from 0.242 to 0.262, with an average of 0.252 per primer pair. The most informative primer combination for distinguishing the genotypes was E-ACC/M-CAT, with the highest values of *R_p* (81.9) and PIC (0.262) (Table 1).

3.2. Population structure

The program STRUCTURE [57,58] was run independently four times, with *K* ranging from 1 to 10, in order to study the structure of the caper population analyzed. The plot of mean posterior probability ($\ln P(D)$) values per clusters (*K*) as well as the ΔK plot based on $\ln P(D)$ values indicated that the most likely number of populations (*K*) was 6 (Fig. 1A and B). Each morphological group of caper, *C. spinosa* subsp. *spinosa* var. *spinosa*, *C. sicula* subsp. *sicula*, *C. orientalis*, *C. ovata* subsp. *ovata*, *C. aegyptia* and *C. zoharyi*, was distinct and clustered into a separate genetic group named c.si, c.sp, c.or, c.ov, c.ae, and c.zo, respectively. The graphic representation of the estimated membership coefficients to the clusters for each accession (at *K* = 6) is shown on Fig. 2. At *K* = 2, *C. sicula* subsp. *sicula* and *C. spinosa* subsp. *spinosa* var. *spinosa* represented by c.si and c.sp respectively, split off from the other species and together formed a separate subpopulation. Increasing *K* to 3, the accessions largely separated into a *C. sicula* subsp. *sicula*–*C. spinosa* subsp. *spinosa* var. *spinosa* group, a *C. orientalis*–*C. ovata* subsp. *ovata* group and a group containing *C. aegyptia*–*C. zoharyi*. The population structure was also assessed individually for each AFLP primer combinations leading to the same clustering (data not shown). Thirty percent of admixed individual was observed in the population; 80%

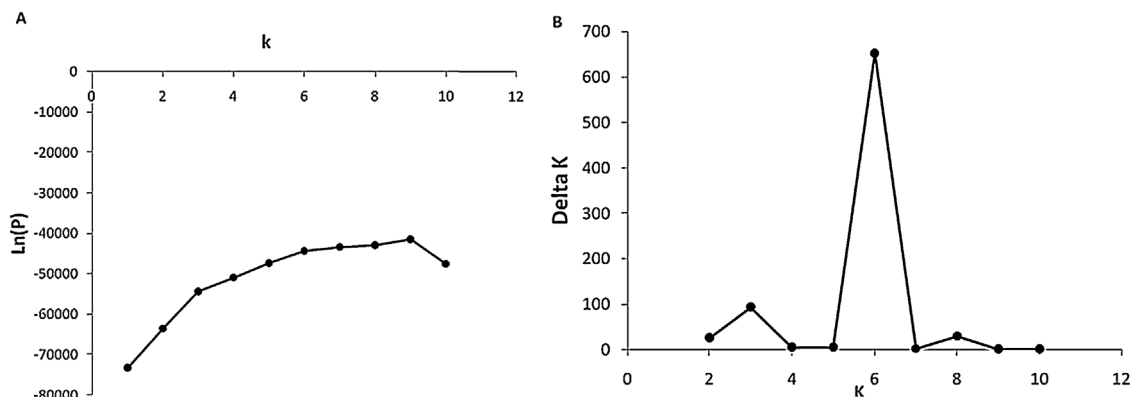


Fig. 1. Plot of mean posterior probability ($\ln P(D)$) values (A) per cluster (*K*), based on 4 replicates per *K*, for *K* ranging from 1 to 10, generated by the STRUCTURE program [57], and delta-*K* analysis (B) of $\ln P(D)$, according to Evanno et al. [59]. A burn-in period of 50,000 and Monte Carlo Markov Chain replicates of 100,000 iterations were used.

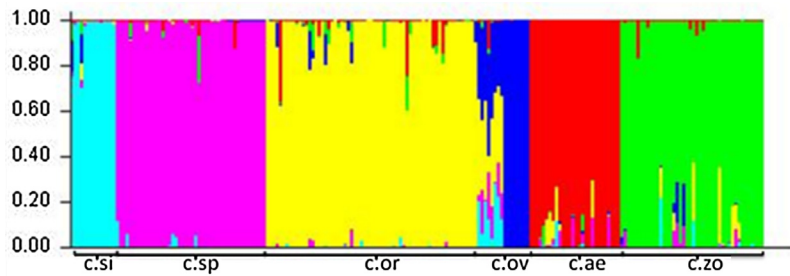


Fig. 2. Proportion of membership coefficient at $K=6$ for 213 Tunisian *Capparis* accessions belonging to 6 species using 636 amplified fragment length polymorphism (AFLP) loci under STRUCTURE software. The different genetic populations, c1, c2, c3, c4, c5 and c6, identified under STRUCTURE were color-coded in light blue, pink, yellow, dark blue, red, and green, respectively. Genotypes are ordered by their morphological similarities to a particular *Capparis* species, c.si, c.sp, c.or, c.ov, c.ae and c.zo correspond to *C. sicula* subsp. *sicula*, *C. spinosa* subsp. *spinosa* var. *spinosa*, *C. orientalis*, *C. ovata* subsp. *ovata*, *C. aegyptia*, and *C. zoharyi* species, respectively.

of them were morphologically identified as *C. ovata* subsp. *ovata*. These hybrids are mixed between *C. sicula* subsp. *sicula*, *C. spinosa* subsp. *spinosa* var. *spinosa* and *C. orientalis* at about 33% each.

The PCoA of the 213 caper accessions showed that the first two axes accounted respectively for 16.19% and 15.19% of the genetic variation, explaining altogether 31.38% of the total variation. The second axis clearly separated the three clusters G1, G2 and G3, while the first axis differentiates the species within clusters G1 and G2. Color-coding of the accessions in the 2-dimensional PCoA plot showed a good correspondence with the population groups obtained from the STRUCTURE analyses (Fig. 3). The presence of intermediate genotypes between clusters confirmed STRUCTURE results of admixed individuals (Fig. 2).

3.3. Analyses of molecular variance and diversity indices

The six populations identified from STRUCTURE analysis were also subjected to analysis of variance (AMOVA) to estimate the percentage of variation among populations and within population. The AMOVA indicated a highly significant ($P < 0.01$) genetic differentiation within the

sampled *Capparis* populations. Fifty percent of the total genetic variance was attributed to the populations based on structure, and the remaining 50% was explained by individual differences within population (Table 2a). In addition, our results determined a high number of specific alleles for each species (population) (Table 3). *Capparis spinosa* subsp. *spinosa* var. *spinosa* presented the highest number of specific alleles (103 AFLP loci) and *C. ovata* subsp. *ovata* presented the smallest number of specific alleles (42 AFLP loci). This result indicated that *C. spinosa* subsp. *spinosa* var. *spinosa* and *C. ovata* subsp. *ovata* were probably the most differentiated and non-differentiated populations, respectively. We also showed that *C. ovata* subsp. *ovata* has the less genetic deviation between accessions ($d = 0.071$), while *C. orientalis* has the highest deviation indices ($d = 0.137$). This indicates that *C. ovata* subsp. *ovata* is the less diverse population, while *C. orientalis* is the most diverse (Table 3).

3.4. Phylogenetic analysis and genetic distances

The dendrogram constructed using UPGMA clustering method separated all the accessions of six species of caper studied into three main clusters; and each species

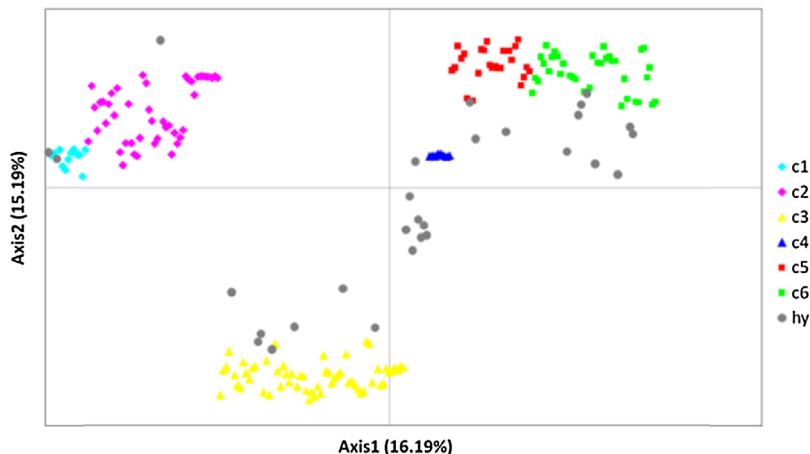


Fig. 3. Principal coordinate plot depicting the six genetic populations of *Capparis* within 213 Tunisian accessions using 636 amplified fragment length polymorphism (AFLP) loci under GenAlex software. The different genetic populations, c1 to c6, identified under STRUCTURE were color-coded in light blue, pink, yellow, dark blue, red, and green, respectively. Genotypes were color-coded according to their membership to each of the six genetic populations. hy, color-coded in gray, referred to admixed individuals according to STRUCTURE results.

Table 2

Analysis of molecular variance (AMOVA) of 213 Tunisian *Capparis* accessions belonging to six genetic populations (a) and 4 bioclimatic stages (b) using 636 AFLP loci performed under GenAlex.

| Source of variation | Degree of freedom | Sum of squares | Variance component | Estimated variation | Percentage of variation | P-value |
|-------------------------------|-------------------|----------------|--------------------|---------------------|-------------------------|---------|
| a. Genetic populations | | | | | | |
| Among populations | 5 | 10742.230 | 2148.446 | 61.98 | 50 | 0.01 |
| Within population | 207 | 12724.624 | 61.47 | 61.47 | 50 | |
| Total | 212 | 23466.854 | | 123.45 | 100 | |
| b. Bioclimatic stages | | | | | | |
| Among bioclimatic stages | 3 | 5573.85 | 1857.95 | 37.91 | 31 | 0.01 |
| Within bioclimatic stage | 209 | 17892.99 | 85.61 | 85.61 | 69 | |
| Total | 212 | 23466.85 | | 123.45 | 100 | |

AFLP: amplified fragment length polymorphism.

Table 3

Diversity indexes within each Tunisian *Capparis* populations and Tajima's Neutrality Test calculated using MEGA software.

| Genetic code ^a | M | S | p_s | Θ | Π | D (P-value) | Specific AFLP bands (number: size – range in bp) | d |
|---------------------------|----|-----|-------|----------|-------|----------------|--|-------|
| c.si | 12 | 219 | 0.232 | 0.077 | 0.081 | 0.298 (0.081) | 62: 69 – 497 | 0.082 |
| c.sp | 45 | 597 | 0.634 | 0.145 | 0.131 | -0.334 (0.131) | 103: 55 – 446 | 0.132 |
| c.or | 60 | 605 | 0.642 | 0.137 | 0.136 | -0.024 (0.136) | 53: 51 – 499 | 0.137 |
| c.ov | 8 | 157 | 0.166 | 0.064 | 0.070 | 0.562 (0.070) | 42: 67 – 480 | 0.071 |
| c.ae | 25 | 466 | 0.495 | 0.131 | 0.107 | -0.728 (0.107) | 82: 70 – 492 | 0.107 |
| c.zo | 37 | 519 | 0.551 | 0.132 | 0.122 | -0.273 (0.122) | 65: 62 – 406 | 0.123 |

M: number of accessions; S: number of segregating AFLP bands; p_s : frequency of allele at the given site; Θ : Watterson theta; D: Tajima's neutrality test; Π : nucleotide diversity; d: estimate of average evolutionary divergence over AFLP fingerprint pairs in same groups.

^a c.si, c.sp, c.or, c.ov, c.ae and c.zo were defined according to STRUCTURE results and correspond to the morphologically distinct species *C. sicula* subsp. *sicula*, *C. spinosa* subsp. *spinosa* var. *spinosa*, *C. orientalis*, *C. ovata* subsp. *ovata*, *C. aegyptia* and *C. zoharyi*, respectively.

constitute a unique subgroup as described in STRUCTURE analysis (Fig. 4). The first cluster, marked G1, grouped the two species *C. sicula* subsp. *sicula* (c1) and *C. spinosa* subsp. *spinosa* var. *spinosa* (c2). The second cluster G2 contained the two species *C. orientalis* (c3) and *C. ovata* subsp. *ovata* (c4). The third cluster G3 grouped the two species *C. aegyptia* (c5) and *C. zoharyi* (c6). In each subgroup (species), all genotypes shared the same ancestor.

Genetic similarity coefficients of the six genetic groups (species) based on Nei's genetic distance is given in Table 4. The high values of genetic similarity coefficients indicated close genetic relationships between populations and the low values indicated remote relationships among populations. The highest similarity value (0.298) was recorded between *C. spinosa* subsp. *spinosa* var. *spinosa* and *C. aegyptia*, while the second highest similarity value (0.286) was found between *C. spinosa* subsp. *spinosa* var. *spinosa* and *C. zoharyi*. In addition, the lowest Nei's genetic distance (0.223) was found between *C. zoharyi* and *C. ovata* subsp. *ovata*, and between *C. aegyptia* and *C. zoharyi*, indicating that *C. zoharyi* and *C. ovata* subsp. *ovata*, as well as *C. zoharyi* and *C. aegyptia*, are the most genetically related species. Similarly, low Nei's distances were also recorded between *C. sicula* subsp. *sicula* and *C. spinosa* subsp. *spinosa* var. *spinosa*, and between *C. orientalis* and *C. ovata* subsp. *ovata* with 0.225 and 0.234 respectively. Furthermore, pairwise N_m values ranged from 0.226 to 0.588 suggesting frequent gene flow among populations. Similar levels of gene flow were recorded between populations within genetic cluster as N_m values between *C. sicula* subsp. *sicula* and *C. spinosa* subsp. *spinosa* var.

spinosa, between *C. orientalis* and *C. ovata* subsp. *ovata*, and between *C. aegyptia* and *C. zoharyi* were 0.522, 0.504 and 0.588, respectively (Table 4).

3.5. Genetic diversity is correlated with geographic distance

Mantel's test in the entire *Capparis* population rejected the null hypothesis as a correlation ($R_{xy} = 0.145$; $P < 0.001$) between genetic and geographic distances was observed among accessions. However, this was not true when the Mantel test was performed for accessions within each cluster individually suggesting that each population is geographic localized (Table 5). Population clustering largely corresponded to climatic zones in Tunisia (Fig. 5). The two species of the G1 cluster, *C. spinosa* subsp. *spinosa* var. *spinosa* and *C. sicula* subsp. *sicula* were mostly (at 83.33%) grown in Saharan bioclimate; the two species of the G2 cluster, *C. orientalis* and *C. ovata* subsp. *ovata*, were frequent at 70% in arid bioclimate; while 95% of the two species of G3 cluster, *C. aegyptia* and *C. zoharyi*, were grown in semi-arid bioclimate. In addition, the major frequencies of hybrid species (92%) were present in northern Tunisia, represented by the subhumid and semi-arid bioclimates. The subhumid bioclimate grouped the highest number of species (5 out of 6). *Capparis spinosa* subsp. *spinosa* var. *spinosa* and *C. orientalis* were the only two species identified in all bioclimatic stages. The analyses showed a significant correlation ($r = 0.956$; $P < 0.0001$) between the number of hybrids and the diversity indices of the bioclimatic stages. A north–south gradient revealed the highest number of hybrids, and

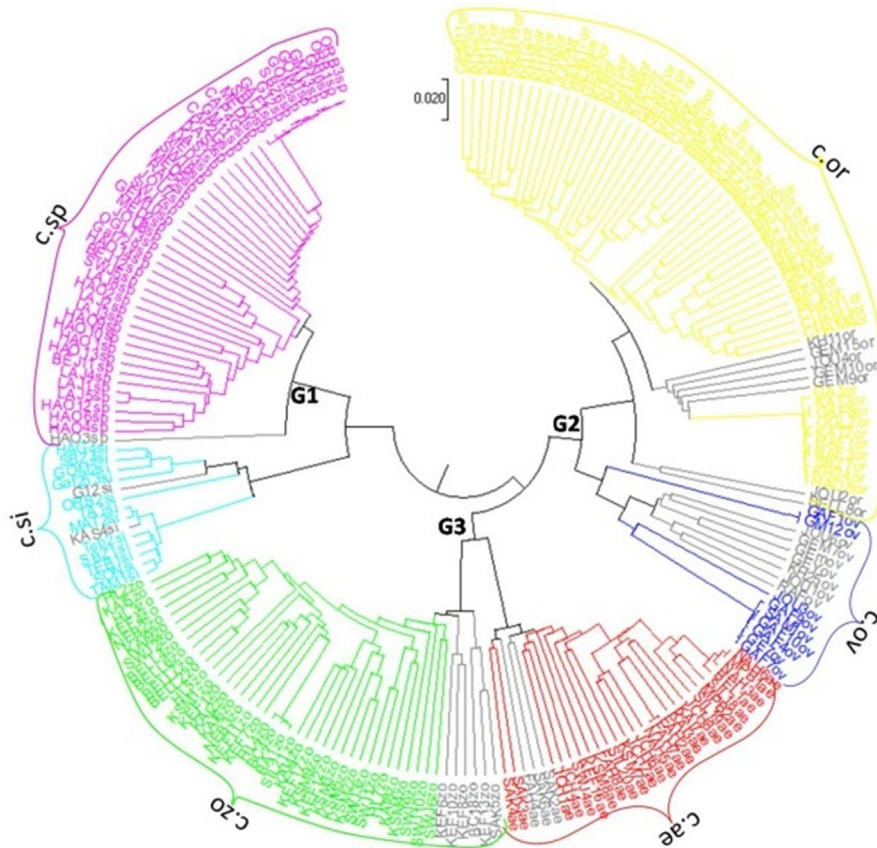


Fig. 4. UPGMA clustering of Nei's genetic distance based on the amplified fragment length polymorphism (AFLP) data of 213 Tunisian *Capparis* accessions performed under Mega software. Genotypes were color-coded in light blue, pink, yellow, dark blue, red and green according to their membership to c1, c2, c3, c4, c5 and c6 respectively. Admixed individuals were color-coded in gray. G1, G2 and G3 correspond to the three main genetic clusters identified. c.si, c.sp, c.or, c.ov, c.ae and c.zo correspond to the morphologically distinct species of *C. sicula* subsp. *sicula*, *C. spinosa* subsp. *spinosa* var. *spinosa*, *C. orientalis*, *C. ovata* subsp. *ovata*, *C. aegyptia* and, *C. zoharyi*, respectively.

Table 4

Pairwise Nei's genetic distances (lower diagonal) and Nm values (upper diagonal) between the *Capparis* populations based on AFLP data.

| c.si | c.sp | c.or | c.ov | c.ae | c.zo | |
|-------|-------|-------|-------|-------|-------|------|
| 0.000 | 0.522 | 0.443 | 0.260 | 0.288 | 0.352 | c.si |
| 0.225 | 0.000 | 0.483 | 0.428 | 0.350 | 0.405 | c.sp |
| 0.253 | 0.273 | 0.000 | 0.504 | 0.408 | 0.502 | c.or |
| 0.226 | 0.249 | 0.234 | 0.000 | 0.374 | 0.476 | c.ov |
| 0.270 | 0.298 | 0.275 | 0.225 | 0.000 | 0.588 | c.ae |
| 0.267 | 0.286 | 0.258 | 0.223 | 0.223 | 0.000 | c.zo |

AFLP: amplified fragment length polymorphism; c.si, c.sp, c.or, c.ov, c.ae and c.zo were defined by STRUCTURE analysis and correspond to *C. sicula* subsp. *sicula*, *C. spinosa* subsp. *spinosa* var. *spinosa*, *C. orientalis*, *C. ovata* subsp. *ovata*, *C. aegyptia* and, *C. zoharyi* species, respectively.

diversity indices were observed in northern Tunisia (represented by the subhumid and semi-arid bioclimates), whereas the genetic diversity drops toward the south (represented by the arid and Saharan bioclimates) (Fig. 6). Our AMOVA analysis revealed that 31% of the total genetic diversity is explained by the bioclimatic stages (Table 2b).

Table 5

Correlation between genetic distance and geographic distance for each of the genetic cluster G1, G2 and G3 of *Capparis* in Tunisia based on a Mantel's test using AFLP data.

| Genetic cluster ^a | R_{xy} | $P(R_{xy-rand} \geq R_{xy-data})$ |
|------------------------------|----------|-----------------------------------|
| All | 0.145 | 0.001 |
| G1 | 0.177 | 0.01 |
| G2 | 0.139 | 0.02 |
| G3 | 0.055 | 0.06 |

AFLP: amplified fragment length polymorphism; R_{xy} : correlation coefficient of Mantel's test; $P(R_{xy-rand} \geq R_{xy-data})$: probability of positive autocorrelation.

^a Genetic clusters G1 to G3 were defined by UPGMA analysis.

3.6. Neutrality test

Tajima's D [63] test was performed to identify populations that do not fit the neutral theory model. The neutrality test of Tunisian *Capparis* sp. displayed significant negative Tajima's D values for five populations,

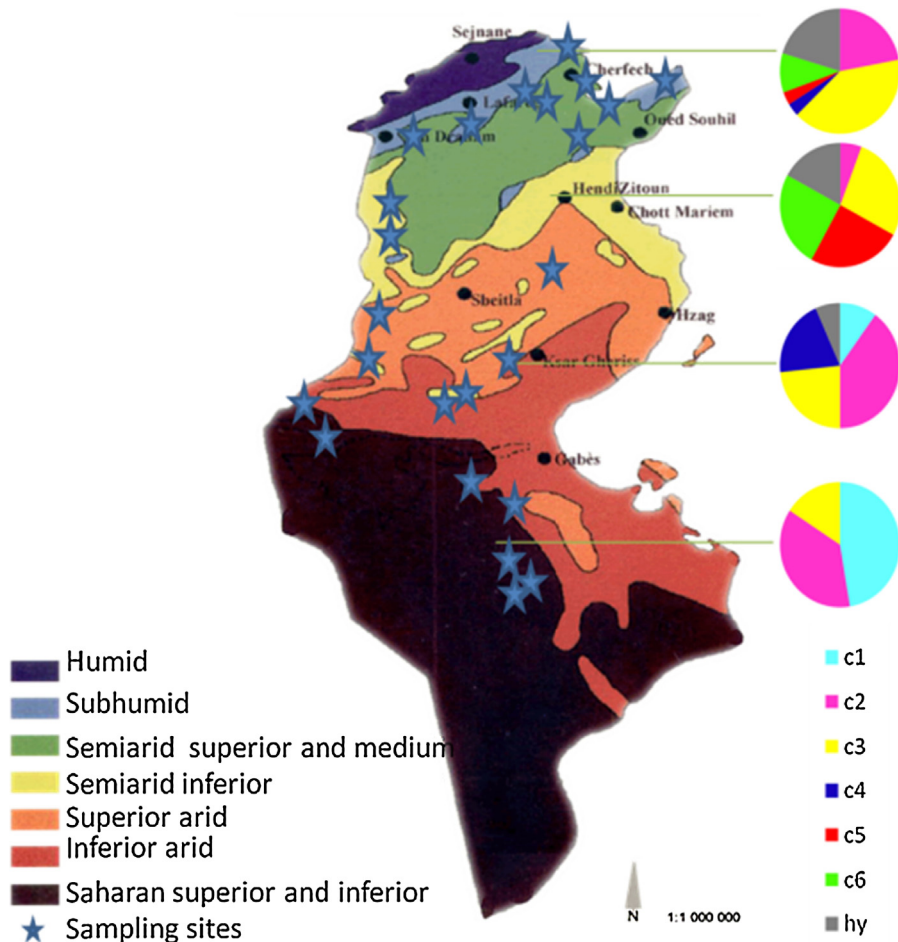


Fig. 5. Geographic structure of 213 *Capparis* accessions over the Tunisian climatic zones showing the differential localization of the six genetic populations as defined by STRUCTURE. The six different genetic populations c1 to c6 were color-coded in light blue, pink, yellow, dark blue, red, and green, respectively. hy, color-coded in gray, referred to admixed individuals according to STRUCTURE results.

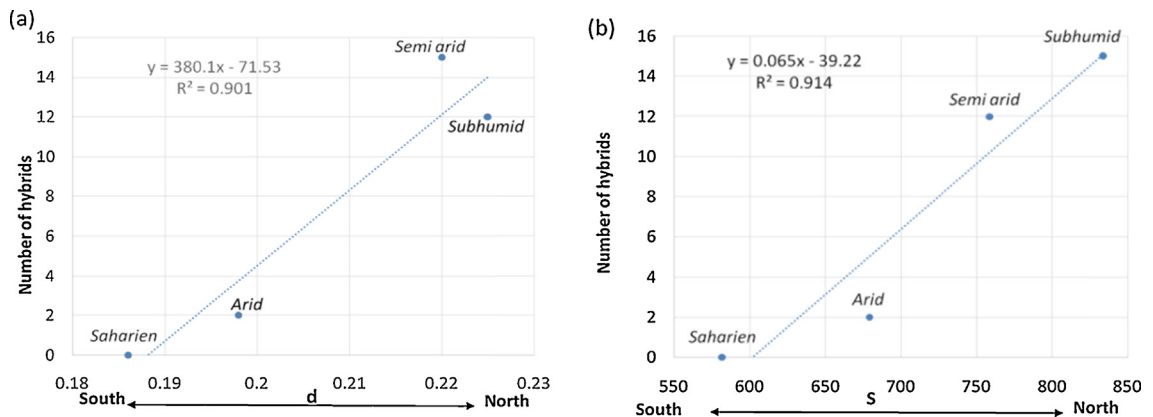


Fig. 6. Correlation between the number of hybrids and the estimate of average evolutionary divergence over amplified fragment length polymorphism (AFLP) fingerprint pairs in same group (a) and the number of segregating AFLP bands across the bioclimatic stages (b). d: the estimate of average evolutionary divergence over AFLP fingerprint pairs in same group; S: number of segregating AFLP bands.

indicating an excess of rare variation, consistent with population growth (Table 3). The population of *C. aegyptia* presented the highest negative value of Tajima's *D* (−0.728), while for *C. orientalis* Tajima's *D* value was the closest to zero (−0.024). For *C. sicula* subsp. *sicula* and *C. ovata* subsp. *ovata*, Tajima's *D* value was positive, indicating balancing selection.

4. Discussion

Caper classification is ambiguous and highly controversial; its population structure has been poorly studied. Various taxonomists have recognized 250 morphologically different species in the genus *Capparis*. Most of the confusion is due to free hybridization of different species and occurrences of intermediate forms. The perplexing condition of the *Capparis* phylogeny has driven many researchers to resolve the classification ambiguities of wild and cultivated forms of *C. spinosa* genotypes using molecular markers. The objective of this study was to genetically characterize 213 *Capparis* sp. accessions sampled from diverse sites in Tunisia in order to study population structure and to correlate the genetic makeup with the morphological description and geographical distribution of *Capparis* species.

The three AFLP primer combinations revealed the same level of polymorphism and diversity indices, and showed the same power to discriminate between the *Capparis* species. Our results support the importance of AFLP analysis where only one primer combination is sufficient for diversity studies. In the literature, very scarce information regarding the genetic diversity of *Capparis* species is known. Our AFLP results revealed a high polymorphism level in the Tunisian *Capparis* genus similarly to previous RAPD [28,29,31,32], and ISSR [29,33,34] studies where the polymorphism among *Capparis* accessions ranged from 48.8% to 98% and from 75.51% to 100%, respectively, with an average of 12.3 polymorphic fragments per primer. The previous AFLP analysis in *Capparis* was performed in 45 accessions from Spain, Morocco, and Syria, and revealed 50% of polymorphic bands and showed that *C. aegyptia* and *C. ovata* are quite separated from the other taxa. The two groups correspond to the populations from Syria as opposed to Morocco and Spain, showing that geographical differences exist [64]. Also, this analysis was performed between samples collected from six sites in Aleppo and Lattakia provinces (Syria) and revealed a certain number of specific alleles for each province and the presence of genetic and reproductive isolation deterrent to gene flow between the two provinces [46]. However, the comparison of our AFLP diversity level in Tunisian *Capparis* populations with previous genetic studies worldwide is difficult. The amount of genetic diversity is related to the marker used and to the *Capparis* population and its size [64].

The present molecular study helped to solve taxonomical ambiguities and the genetic relationship among caper accessions in Tunisia. Out of the 636 polymorphic fragments generated, 407 were species-specific. Our STRUCTURE study was able to distinguish six populations of *Capparis* in Tunisia with their AFLP fingerprint,

corresponding to the six morphologically characterized species *C. orientalis*, *C. ovata* subsp. *ovata*, *C. aegyptia*, *C. zoharyi*, *C. sicula* subsp. *sicula* and *C. spinosa* subsp. *spinosa* var. *spinosa*. This genetic result was in line with the morphological classification of Inocencio et al. [8], Le Floc'h et al. [48], and Yousfi [49]. The results obtained in this study both at the individual and population levels indicated that *C. zoharyi* constitutes a separate gene pool from the other species, especially from *C. aegyptia* and should be considered as a species by itself, contradicting with Fici [10] morphological classification. In our study, *C. zoharyi* presents 65 specific alleles and is genetically as distant from *C. aegyptia* as *C. sicula* subsp. *sicula* is distant from *C. spinosa* subsp. *spinosa* var. *spinosa* and *C. ovata* subsp. *ovata* is distant from *C. orientalis*. In addition, our phylogenetic analysis clarified the relationship between the six species and suggested their classification into three main genetic clusters. The first cluster (G1) grouped the two species *C. spinosa* subsp. *spinosa* var. *spinosa* and *C. sicula* subsp. *sicula*. These species have a procumbent habit, with persistent stipules that are not thorny, but are slender and curved, and grow mainly in the Saharan bioclimate. The second cluster (G2) contained the two species *C. orientalis* and *C. ovata* subsp. *ovata*, having a pendulous habit, deciduous stipules that are not thorny, but are slender and straight, and growing mainly in the subhumid bioclimate. The third cluster (G3) grouped the two species *C. aegyptia* and *C. zoharyi* that have an erect habit, with evergreen stipules that are spiny, wide and crooked and growing mainly in the semi-arid bioclimate. The geographic distribution pattern of these species in Tunisia and the spatial proximity between species within genetic clusters probably explain the similar gene flow level found in our study between *C. sicula* subsp. *sicula* and *C. spinosa* subsp. *spinosa* var. *spinosa*, between *C. orientalis* and *C. ovata* subsp. *ovata*, and between *C. aegyptia* and *C. zoharyi* within G1, G2 and G3 genetic clusters, respectively. In addition, this study revealed that accessions from G2 and G3 are more genetically related compared to accessions from G1. These genetic results refute again Fici's [10] morphological classification where *C. aegyptia* (G3 here) was shown to be more closely related to *C. spinosa* subsp. *spinosa* var. *spinosa* and *C. sicula* subsp. *sicula* (G1), because of its persistent thorns, than to *C. orientalis* and *C. ovata* subsp. *ovata* (G2 here). Our results also contradict the previous work by Saadaoui et al. [65] reporting that *C. spinosa* subsp. *spinosa* was found in northern Tunisia (subhumid and semi-arid bioclimatic zones), whereas subsp. *rupestris* (*C. orientalis*) had a larger geographical distribution from subhumid to Saharan regions. On the other hand, the present study revealed that *C. spinosa* is commonly grown in the wild in diverse geographical and climatic regions in Tunisia contradicting Inocencio et al. [8] studies reporting that most known populations of *C. spinosa* were cultivated. In addition, our study did not support Inocencio et al. [64] hypothesis on the hybrid origin of *C. spinosa* between *C. orientalis* and *C. sicula*. Previous studies considering *C. aegyptia* as a varietal rank of *C. spinosa* (*C. spinosa* var. *aegyptia* (Lam.) Boiss) [4,61,66] were also refuted here.

This study was the first to cover most of the geographical and ecological areas and the various *Capparis* species growing in Tunisia. The AFLP polymorphism found between species suggests high variations at the genomic level that might have been accumulated during speciation events in *Capparis* as is evident from its morphological diversity. In addition, genotypes within species were genetically diverse; 50% of the total genetic diversity is observed within species. According to Khouildi et al.'s [50] results, the genetic variation found could be more likely related to environmental factors. Our analysis revealed that 31% of the total genetic diversity is explained by the bioclimatic stages. Ecological adaptations of *Capparis* to different environments could have been driven by isolation. The particularly high level of genetic variation, the negative value of Tajima's *D* within species, the population structure, and the presence of geographical patterns in diversity suggest several hypotheses regarding the population history of *Capparis* species. First, *Capparis* did not recently colonized Tunisia, and we confirm here its endemism. Second, *Capparis* species may be undergoing population expansion and may have large population sizes. Third, our results revealed intermediate genetic diversity levels in *C. aegyptia* and *C. zoharyi* while *C. sicula* subsp. *sicula* and *C. ovata* subsp. *ovata* had low diversity indices. Assuming a same evolutionary rate in all species, we can suggest that through speciation, the divergence between *C. aegyptia* and *C. zoharyi* occurred earlier than the divergence between *C. sicula* subsp. *sicula* and *C. spinosa* subsp. *spinosa* var. *spinosa*, and the divergence between *C. orientalis* and *C. ovata* subsp. *ovata*. Fourth, *Capparis*'s first settlement occurred in Northern Tunisia and then migrated during speciation across the country and colonized the southern regions. Subhumid bioclimate of Northern Tunisia is the most diversified grouping the three genetic clusters and five species out of six and is probably similar to the origin climate of the ancestral form. This genetic study also revealed that hybridization events are more frequent in the northern area, where all the genetic clusters are in sympatry. The region of greatest diversity was shown to be the origin area for many other wild species in plants [67–70]. Furthermore, from our phylogenetic results, we suggest *C. spinosa* subsp. *spinosa* var. *spinosa* (G1 genetic cluster), a species reported in all bioclimate (from subhumid to Saharan) and with high diversity indices, as the closest species to the ancestral form that generated the different *Capparis* species. *Capparis spinosa* subsp. *spinosa* var. *spinosa* was probably the first species to be differentiated during speciation in *Capparis* and had enough time to evolve and adapt to different geographic areas. In fact, Renfrew [71] mentioned that *C. spinosa* var. *spinosa* was founded in fossils seeds from Iraq 5800 years before Christ. A worldwide study is necessary to confirm our hypothesis on *Capparis* evolution, speciation events and adaptation to geographic areas.

5. Conclusion

In conclusion, our study showed that AFLP analysis is an efficient method to investigate the genetic diversity and

the relationship between species in *Capparis*. A total of six morphologically distinct species of caper were genotyped in Tunisia using 3 *EcoRI*–*MseI* AFLP primer combinations. Data analysis revealed a high degree of polymorphism allowing the distinction of the six species and their grouping into three main genetic clusters emphasizing the existence of recognizable genetic similarity within species and genetic heterogeneity between them. These three main clusters seem to be adapted to different geographic areas in correlation with bioclimate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.crvi.2016.09.001>.

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