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Genetic Structure of Mediterranean Fruit Fly (*Ceratitis capitata*) Populations from Moroccan Endemic Forest of *Argania spinosa*

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ABSTRACT

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Diptera: Tephritidae), widely distributed around the World, is regarded as the most important pest of agricultural resources. The genetic structure of this species remains unexplored at microgeographical level, specifically in the African countries. In Morocco, the endemic Argan forest of 800,000 hectare is considered as the world main refuge of medfly and probably a continuous source of medfly infestations and invasions to neighbouring regions. In this study, RAPD was used to analyze the genetic structure of Moroccan medfly populations, collected from distant regions and at different altitudes within the Argan forest, a Kenyan medfly population and a Moroccan medfly laboratory strain. Based on the analysis of 169 RAPD loci, the population genetics parameters were estimated and two dendrograms (UPGMA & neighbor-joining clustering) were constructed. The genetic diversity parameters (Shannon information index; total number & percentage of polymorphic loci) were very high in Moroccan medfly populations (P = 81%; 0.112 ± 0.015 < I < 0.175 ± 0.020), particularly in populations sampled at higher altitudes (I = 0.175 ± 0.020). However, these parameters were slightly lower compared to Kenyan ones (P= 89%; I = 0.227 ± 0.020). The AMOVA and F-statistics showed the occurrence of important genetic differentiation between Moroccan medfly populations (F_{ST} = 0.448). Furthermore, our data indicate that the genetic structure of Moroccan medfly populations was determined predominantly by altitude. Inferences about the historical colonization and establishment of medfly populations in this country were discussed.

Key Words: Genetic diversity; Genetic differentiation; Altitude; *Ceratitis capitata*; RAPD; Argan forest

INTRODUCTION

The Mediterranean fruit fly is a polyphagous fruit fly attacking over 300 different fruits, vegetables and nuts and causing important economic losses to the cosmopolitan agriculture (White & Elson-Harris, 1992). The medfly is widely distributed throughout most of the African continent, the Mediterranean region, the central and the southern of American continent and parts of Australia (Gasperi *et al.*, 2002). It is an invasive species, characterized by its ability to invade and colonize a wide variety of agricultural areas, particularly with tropical, sub-tropical and temperate climates (De Meyer *et al.*, 2008). To achieve effective medfly management, the medfly biology, behaviour, ecology and genetics have been extensively studied (Meats & Smallridge, 2007; Stratikopoulos *et al.*, 2008; Robinson & Hooper, 1989; Aluja & Norrbom, 2001). However, the population genetics of this species is still poorly studied at micro-geographical level.

The genetic structure of geographically distant medfly populations has been analyzed using allozyme, mitochondrial DNA, RAPD, intron sizes and microsatellites markers (Baruffi *et al.*, 1995; Gasparich *et al.*, 1997; Gomulski *et al.*, 1998; Malacrida *et al.*, 1998; Kourti, 2002; 2004a & b; Gasperi *et al.*, 2002; Bonizzoni *et al.*, 2004; Malacrida *et al.*, 2007; Barr, 2009). Notable genetic variability and higher average heterozygosity have been found within African medfly populations compared to Mediterranean ones. Also, these studies suggest that *C. capitata* has originated from South-Eastern Africa and spread historically to the Mediterranean regions, then to the Central and Southern America and the Hawaiian Islands, where it became established within the last 200 years. The medfly invasion of the Mediterranean region, particularly the African part, need to be re-examined profoundly because geographic barriers (large desert & higher mountains) would have hindered the medfly invasions from the South-Eastern to the Northern regions of Africa.

In Morocco, the medfly is widely distributed and its management relies only on the insecticide uses. Besides the environmental, ecological and human health problems caused by pesticides, they were not able to reduce significantly such large medfly infestations. The situation is even more complicated since the medfly survives in

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large forests of Argan trees. The Moroccan endemic forest of 21 millions Argan trees (800,000 hectares) is regarded as the World main refuge of medflies (Debouzie & Mazih, 1999). The genetic structure of this Moroccan medfly 'reservoir' may help us to gain a better understanding of the genetic differentiation of *C. capitata* as well as the factors involved in the maintenance and spread of the medfly populations in the Mediterranean region. Accordingly, this paper aims to analyse the micro-geographical genetic variation among medfly population from the Moroccan endemic forest of *Argania spinosa*.

MATERIALS AND METHODS

Insect sampling: Moroccan medfly samples were collected from distant regions and at different altitudes in the Argan forest during the summer of 2007 (Table I; Fig. 1). The medfly adults were obtained from pupae, which emerged from the infested fruits. To attempt to take a random sample and avoid excess of consanguinity, infested fruits were collected from large number of Argan trees, separated from each other by hundred of meters. The medfly samples were preserved in 95% ethanol or stored at -20°C.

The medfly laboratory strain is being reared in our laboratory for more than 30 generations and has originated from medflies, collected from the Moroccan Argan fruits. The Kenyan medfly population was sampled by Dr. Maxwell Billah (ICIPE), preserved in ethanol and sent to our laboratory in 2006. The *Bactrocera oleae* (olive fly) sample was obtained from Marrakech region and preserved at -20°C.

DNA extraction and RAPD genotyping: Genomic DNA was extracted from single medfly following the method described by Aljanabi and Martinez (1997) with minor modifications. The RAPD-PCR reactions were conducted in a total volume of 25 µL containing 2 mM $MgCl_2$, 1x GoTaq buffer, 0.12 mM dNTP, 0.01 mM primer, 1 unit of Go Taq DNA polymerase (Promega) and 50 ng of DNA. The reaction mixtures were run on the same thermocycler according to the following scheme: (a) 2 min 30 sec denaturation at 94°C for the first cycle; (b) 1 min denaturation at 94°C, 1 min annealing at 35°C and 2 min elongation at 72°C for 40 cycles and (c) an additional 8 min elongation at 72°C for the last cycle. RAPD products were separated by electrophoresis on 1.2% agarose gels. The molecular sizes of the amplified DNA bands were estimated using 1 kb and 100 bp ladder (Promega). Forty RAPD primers (OPC & OPE kits from Operon) were tested and only five RAPD primers were selected for our experiments.

Data analysis: Each individual (n =188) was examined by using each of the five selected 10-mer primers. To score RAPD markers, only unambiguous bands were analyzed as RAPD loci. Data from the RAPD banding patterns were coded in a binary data matrix.

The total numbers of amplified bands and the polymorphic percentages were calculated for each population using TFPGA software. The intra-population genetic diversity was assessed using two indices: (i) heterozygosity Means (He & UHe) and (ii) Shannon diversity Index (I). All these indices were calculated using GENALEX 6.1 software.

AMOVA was used to calculate the variance in RAPD banding patterns of 144 medflies. The Medflies from the laboratory strain and the olive fly samples were excluded from AMOVA calculation. The F_{ST} were obtained by applying Weir and Cockerham's methods of calculating Wright's F statistics. All calculations were performed using the ARLEQUIN 3.0 software.

RAPDs data was used to construct two dendrograms following two methods: UPGMA and neighbor-joining (NJ). Nei's genetic distances were calculated between each pair of the 9 populations using the Binary data. The genetic distance matrix was used to generate a phylogenetic dendrogram using UPGMA. Consistency of tree was checked by a bootstrap value of 1000 at 95% confidence intervals using TFPGA v.1.3.

The genetic similarity matrix of 188 fly was estimated using Jaccard's coefficient and was run on SAHN using the NJ clustering algorithm to generate dendrograms. The fit-goodness was measured using 1000 permutations and provided a cophenetic correlation value (r). All computations were performed using the NTSYSpc 2.1 package.

RESULTS

Screening of RAPD primers and identification of RAPD polymorphism: Five primers (OPC-1, OPC-2, OPC-7, OPC-8 & OPC10) were selected from forty primers based on the polymorphism, intensity and reproducibility of the bands generated (for representative band patterns, see Fig. 2).

These five primers were used in RAPD-PCR reactions containing genomic DNA from each individual of Moroccan and Kenyan medfly populations (7 populations, n = 144), one medfly laboratory strain (n = 24) and the natural olive fly population (n=20). The Moroccan medfly populations were sampled from six geographically distant regions of the Argan forest and at different altitudes (Table I; Fig. 1). The olive fly, *B. oleae* was used as an out-group in these experiments. The five primers generated a total of 169 reliable RAPD loci, with sizes ranging from 0.3 to 3.8 kb (Table II). Out of these, 132 bands (78%) were polymorphic across 188 individuals of *C. capitata* and *B. oleae*. The total number and the percentage of polymorphic loci of the medfly populations were not modified (133 & 82%), if the *B. oleae* samples and the medfly laboratory strain were excluded from our analysis.

The total number of bands scored per primer ranged

Table I: Sampling sites, codes and altitudes of areas where *Ceratitis capitata* and *Bactrocera oleae* (outgroup species) were collected for this study. In the last column, numbers indicate the sampling localities as shown in Fig. 1

Species	Locality	Abbreviation code	Number of individuals	Altitude (m)
Ceratitis capitata	Ait Meloul (2)	MEL	20	36-48
C. capitata	Amskroud (3)	AMS	20	195-227
C. capitata	Immariden (4)	IMA	20	600-700
C. capitata	Tizi n'test (5)	TIZ	24	760-1,200
C. capitata	Essaouira (1)	ESS	20	4-100
C. capitata	Berkane (6)	BER	20	250-300
C. capitata	Nairobi-Kenya	KEN	20	
C. capitata	Laboratory strain	Lab	24	
Bactrocera oleae	Marrakech	BAC	20	

Table II: RAPD primers used in the study and their sequences, the number of amplified DNA fragments they generated and the approximate sizes in base pairs of scored markers

Primer	Nucleotide Sequence 5' to 3'	Number of amplified fragments	Average of amplified fragments/fly	Fragments size (bp range)
Opc1	5'-TTCGAGCCAG-3'	36	4.01	310-3780
Opc2	5'-GTGAGGCGTC-3'	31	5.60	400-2710
Opc7	5'-TTAGTGAGTA-3'	31	4.09	460-3600
Opc8	5'-TGGACCGGTG-3'	34	6.56	390-2930
Opc10	5'-TGTCTGGGTG-3'	37	5.22	280-3660
Average		34	5.10	
Total		169	25	280-3780

Table III: Analysis of molecular variance (AMOVA) for RAPD variation and F-statistics of genetic differentiation among *Ceratitis capitata* populations. P-values represent the probability of obtaining a more extreme component estimate by chance alone (calculated after 10000 permutations)

Source of variation	d.f.	Sum of squares	Variance components		Fixation Indices
			Absolute	%	
Among groups	5	991.23	5.35*	28.71	F_{CT} = 0.29
Among populations within groups	1	70.20	2.99*	16.07	F_{SC} = 0.22
Within populations	137	1409.42	10.29*	55.21	F_{ST} = 0.45
Total	143	2470.85	18.63		*P-value < 10^{-5}

d.f.: degree of freedom

Table IV: Pairwise F_{ST} values (lower diagonal) and gene flow estimates (N_m, upper diagonal) among six Moroccan populations and one Kenyan population of *Ceratitis capitata.* Nm is the effective number of migrants per generation. Abbreviations are as indicated in Table I

Populations	MEL	AMS	IMM	TIZ	ESS	BER	KEN
MEL	*****	0.682	0.576	0.209	0.357	0.608	0.376
AMS	0.268	*****	0.527	0.194	0.349	0.435	0.425
IMM	0.303	0.322	*****	0.231	0.353	0.418	0.397
TIZ	0.544	0.564	0.519	*****	0.176	0.189	0.279
ESS	0.412	0.417	0.415	0.587	*****	0.322	0.363
BER	0.291	0.365	0.374	0.570	0.437	*****	0.388
KEN	0.399	0.370	0.386	0.473	0.408	0.392	*****

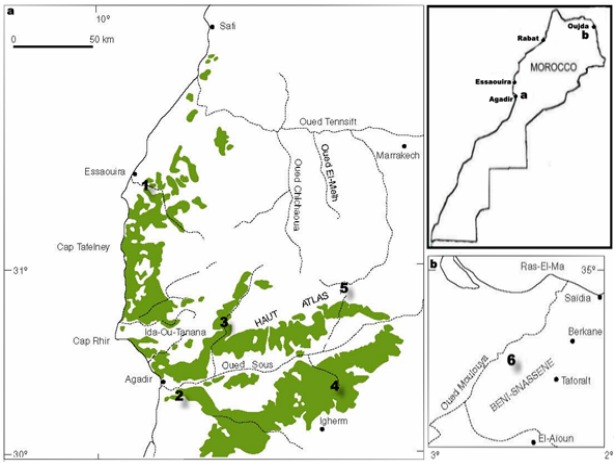
from 31 to 37, with an average value of 34 bands per primer (Table II). The selected primers revealed different numbers of RAPD loci in different medfly populations and very important genetic variability levels. The number of polymorphic fragments generated by each primer also varied from 23 (OPC-2) to 31 (OPC-10), with an average of 26 polymorphic fragments per primer.

RAPD variation within populations of *C. capitata*: The total number and the percentage of polymorphic bands at a frequency superior or equal to 5%, the number of population-specific RAPD loci, the Shannon information index and the heterozygosity were calculated for each population.

The total number and the percentage of shared and specific RAPD loci varied according to the geographic origin and the habitat of medfly populations (Fig. 3). The Moroccan medfly populations contained large numbers of polymorphic loci, but less than the Kenyan one (Fig. 5). The total number of RAPD bands for each population ranged from 90 to 51, whereas the total number of polymorphic ones varied between 80 for Kenyan medfly population and 35 for Moroccan olive fly population (Fig. 5).

The five selected primers generated 13, 10, 10, 5 and 5 population-specific bands respectively with OPC1, OPC7, OPC10, OPC2 and OPC8; a total of 43 population-

Fig. 1: Map of Morocco showing the collection sites of *Ceratitis capitata* populations. The code numbers of the six Moroccan medfly populations are given in Table I



specific bands. The highest number of specific RAPD bands (14 specific loci) was identified in the Moroccan medfly samples from Tizi n'test, compared to other Moroccan and Kenyan medfly populations (Fig. 3 & 4).

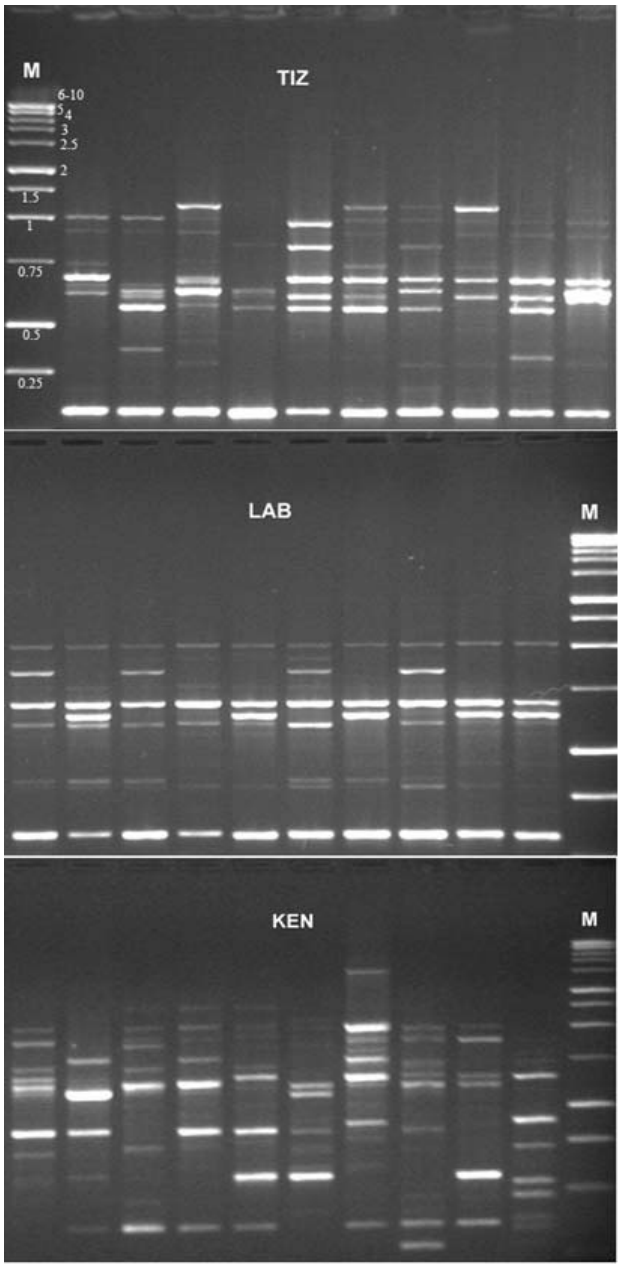
As the total number of RAPD bands was not identical in different population (90 to 51), the percentage of polymorphic bands values was calculated to verify accurately the population polymorphism. The percentages of Polymorphic Bands, estimated at 95% level, ranged from 90% for the Kenyan population to 65% for Moroccan olive fly population (Fig. 6). Both the total number and the percentage of polymorphic bands indicated clearly that the Kenyan medfly population was the most polymorphic one, followed by Imariden medfly population and the other ones.

The Shannon information index (I) was calculated on the basis of 169 RAPD loci, whereas the heterozygosity (He) and unbiased heterozygosity (UHe) were estimated using only 108 RAPD loci. The other 61 RAPD loci were excluded from our analysis, because their overall frequencies of band presence were higher than 1-3/N (where N is the number of individuals sampled) and consequently, will bias the allele frequency.

The heterozygosity average varied from 0.323 to 0.232 within the eight medfly populations (Fig. 7). Among natural medfly populations, the Imariden one showed the highest level of estimated heterozygosity (0.278) over 38 polymorphic loci, followed by Tizi n'test with 0.271. The heterozygosity values of the Moroccan medfly populations and the Kenyan population were not significantly different ($p>0.2$). Because the correlation between the heterozygosity and the number of polymorphic loci is not strong ($r^2 = 0.0373$); the average heterozygosity seems to characterize accurately the population structure.

The genetic diversity average using the Shannon information index was 0.145. The Shannon index ranged from 0.108 for *B. oleae* population to 0.227 for Kenyan

Fig. 2: Example of RAPD band profiles of *Ceratitis capitata* from the Tizi n'test population (TIZ), the Kenyan population (KEN) and the Laboratory strain (LAB) using primer OPC10. Each lane contains amplification products for an individual medfly. M is the 1-kb ladder molecular weight standard, the sizes are in kbp



medfly population (Fig. 7), the latest was significantly different from the other populations ($2x10^{-6} < p < 4x10^{-2}$). Among the Moroccan medfly populations, the Tizi n'test one showed the highest Shannon information index 0.175 followed by the Imariden population 0.169 (Fig. 7). These Shannon indexes were not found related to the latitudinal origin of the Moroccan medfly populations but separated the nine populations on four homogeneous groups ($5x10^{-6} < p < 4x10^{-2}$) depending apparently to altitude more than

Fig. 3: Summary of RAPD loci numbers and frequency for each analyzed populations of *C. capitata* and *B. oleae*. No. Bands: Total number of RAPD Bands scored per population; Freq: Frequency; Lcomm Bands: shared bands within the population. Refer to Table I for population abbreviations

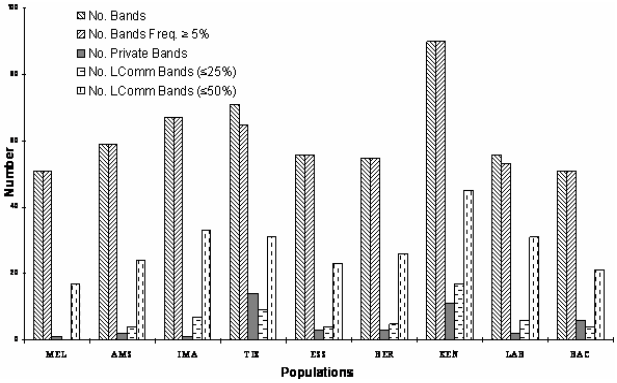
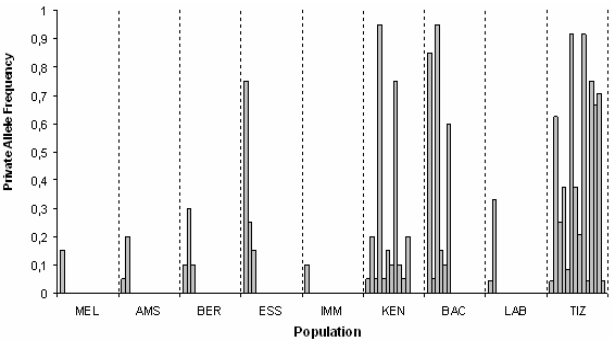


Fig. 4: Frequency of RAPD private bands generated with the five primers OCP1, OCP 2, OCP 7, OCP 8 and OCP 10. Refer to Table I for population abbreviations



geographic space (Fig. 7). Populations with the highest Shannon indexes also exhibited the highest number of polymorphic loci (Fig. 5). The Moroccan Medfly populations (0.175) were less diverse than the Kenyan one (0.227).

RAPD polymorphism between populations of *C. capitata*: The Medfly genetic differentiation was analyzed using AMOVA, the F_{ST} and UPGMA and NJ dendrograms. Assembling the 7 medfly populations into 5 to 4 groups according to their geographical origin and altitude, the AMOVA analysis indicated that 55.2% of the molecular variance among medfly individuals was found within population (Table III), whereas 28.71% was due to the differences among groups and the other 16.07% to variance between populations. The random permutation test revealed that the variance components were all highly significant ($p<10^{-5}$) according to F statistics for all hierarchical levels. The modification of every single parameter (Number of population, selected loci, Hypothetic AMOVA structure & individuals' exclusion) did not change the molecular variance values. When the Shannon index was used for partitioning genetic diversity variance among medfly individuals, 62% of the variance

Fig. 5: Number of polymorphic and monomorphic bands generated with RAPD primers (OCP1, OCP2, OCP7, OCP8 and OCP10) in *C. capitata* and *B. olea* populations. The RAPD locus is considered polymorphic when its frequency is superior to 5% in all populations. Refer to Table I for population abbreviations

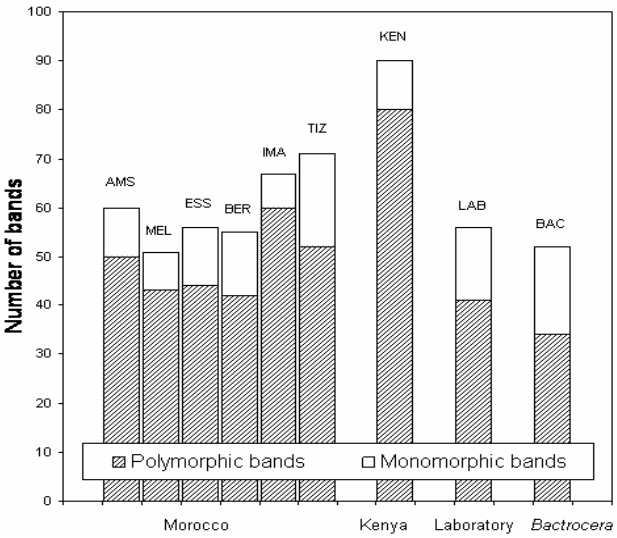
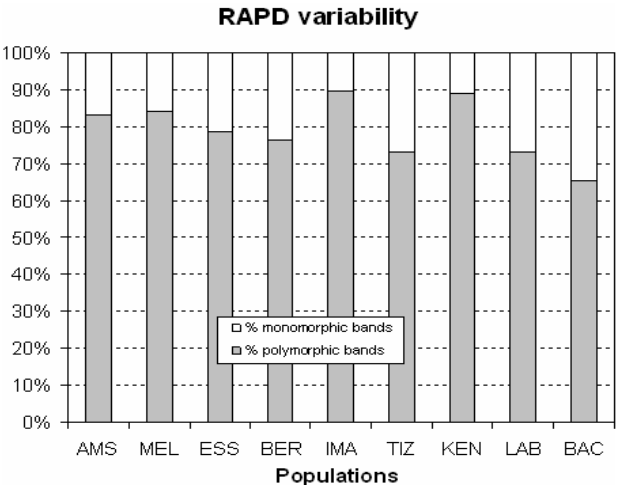


Fig. 6: Percentage of polymorphic and monomorphic bands generated with RAPD primers (OCP1, OCP2, OCP7, OCP8 and OCP10) in *C. capitata* and *B. olea* populations. The RAPD loci are considered polymorphic when its frequency is superior to 5% in all populations. Refer to Table I for population abbreviations



was found within medfly population, whereas the other 38% was attributable to variance between subpopulations. The same data were obtained either with medfly individuals of the six Moroccan medfly populations alone or in addition to the Kenyan one.

The F_{ST} adapted by Lynch and Milligan to dominant markers, was used to examine the overall level of genetic divergence among populations. The F_{ST} of the Medfly populations is 0.444. This data implies that very important genetic differentiation has occurred between the medfly populations. If the Kenyan medfly samples were not included in the calculus, the F_{ST} value increases slightly to

Fig. 7: Genetic diversity statistics of seven medfly populations (MEL; AMS; IMM; TIZ; ESS; BER; KEN), a medfly laboratory strain (LAB) and an olive fly population (BAC) using five RAPD primers. He: Heterozygosity (First Bar); UHe: Unbiased Heterozygosity (Second bar); I: Shannon's diversity index (Third bar). The He, UHe or I values are statistically different when indicated by distinctive alphabet (a, b, c or d)

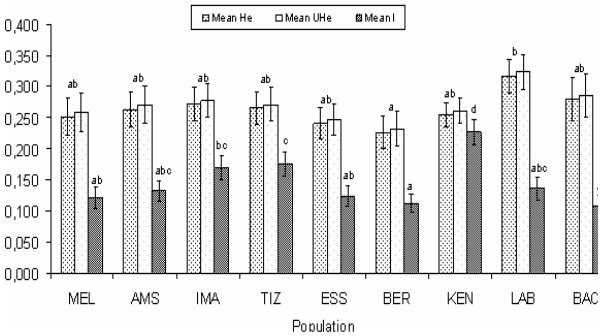
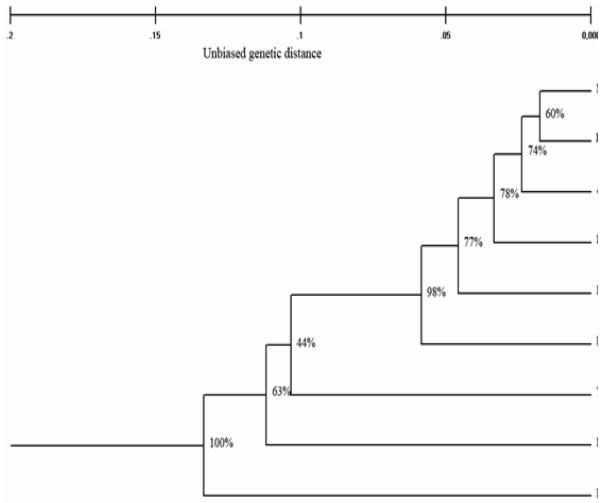


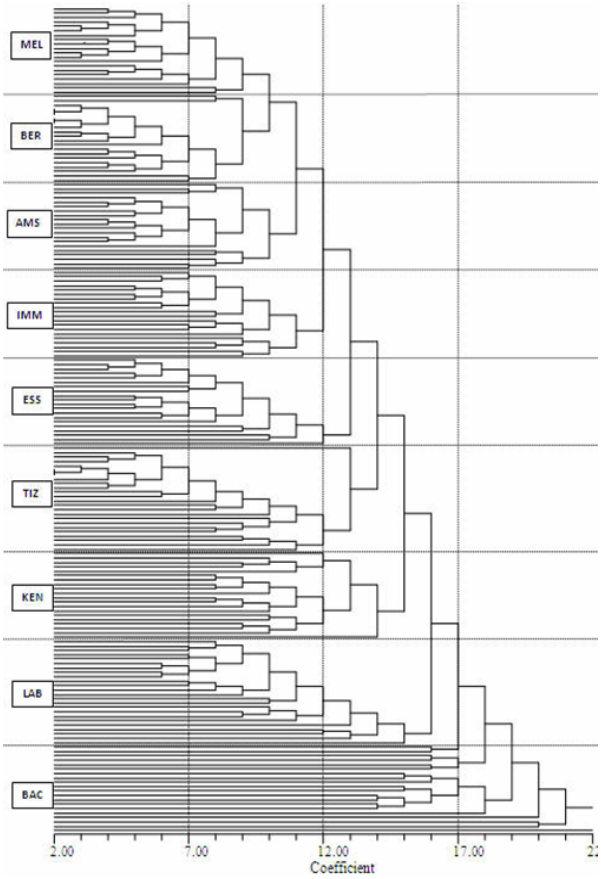
Fig. 8: UPGMA dendrogram based on Nei's genetic distance between six Moroccan medfly populations, a Kenyan medfly population, a medfly laboratory strain and an olive fly population (out-group). Numbers at the internodes indicate all the bootstrap percentage values based on 1,000 replications. Refer to Table I for population abbreviations



0.464. Pairewise F_{ST} values/gene flow (N_m) varies from 0.268/0.682 (Ait Meloul–Amskroud) to 0.587/0.176 (Essaouira–Tizi n'test) and were all statistically significant at a level of 5% (Table IV). These F_{ST} values and the genetic variation between Moroccan medfly populations showed clearly that the genetic structure is determined predominantly by altitude.

To analyze the genetic relationships between medfly populations, two dendrograms were constructed using UPGMA and NJ. UPGMA clusters separately the nine populations (Fig. 8). The medfly Tiz n'test population, sampled from higher altitude, is grouped independently from the other medfly population. The third node shows a lower percentage of bootstrap replication score (44%) after 1000 bootstrap resampling of the input data. The NJ method

Fig. 9: Neighbour-joining tree based on inter-individual genetic similarity of RAPD patterns from six Moroccan medfly populations, a Kenyan medfly population, a medfly laboratory strain and an olive fly population (a total of 188 individuals). The olive fly population is used as an out-group. The correlation coefficient calculated by Mantel test (r = 0.93), indicates the goodness fit of this phenogram. Refer to Table I for population abbreviations



represents individual phenetic relationships between 188 flies from the 9 populations (Fig. 9). The dendrogram was statistically stable by a high cophenetic correlation value (r = 0.93) and clustered the flies of each population altogether. Both UPGMA and NJ dendrograms clustered separately: (i) *C. capitata* from *B. oleae*; (ii) Laboratory medfly strain from medfly populations and (iii) every medfly population alone.

DISCUSSION

The genetic structure of medfly populations has been analyzed in earlier studies at macrogeographical and microgeographical levels (Baruffi *et al.*, 1995; Gasperi *et al.*, 2002; Bonizzoni *et al.*, 2004; Malacrida *et al.*, 2007; Barr, 2009). At the macrogeographical level, Gasperi *et al.* (2002) have suggested a separation of medfly populations into three main groups: (i) ancestral populations from sub-saharan mainland Africa, (ii) ancient populations from the Mediterranean basin and (iii) new populations from America and the Pacific region. At the microgeographical level,

studies have been interested mainly on the new populations from California, Florida, Argentina and Australia (Meixner *et al.*, 2002; Silva *et al*., 2003; Bonizzoni *et al.*, 2004; Lazavecchia *et al.*, 2008) and two Mediterranean countries; the Spanish and the Greek medfly populations (Ochando *et al*., 2003; Kourti, 2004b). The geographic location of Morocco and its forestry may have participated historically to medfly invasion of the Mediterranean region. Morocco is located at the North of Africa (between the sub-Sahara & Europe) and at the southern part of the Mediterranean region and contains large endemic forests of *Argania spinosa*, which were highly attractive to *Ceratitis capitata*. Therefore, the molecular analysis of the Moroccan medflies at the microgeographical level will help us to gain more information about its genetic structure, diversity and differentiation. To our knowledge, Moroccan medfly population has been included in studies at macrogeographical level, but it has been sampled only from one region at the western coast of Morocco (Baruffi *et al.*, 1995; Malacrida *et al*., 1998). In this paper, the Moroccan medfly populations have been sampled from different regions either geographically distant (from 50 km to 1200 km) or at different altitude (from 40 to 1200 m above sea level). It should be noted that the sampling has included only the medfly populations from the endemic Argan forest in order to exclude any population differentiation caused by host specificity. The genetic structure of this Moroccan medfly 'reservoir' has been analyzed using the RAPD markers. The different approaches used for data analysis produce mainly similar values of genetic diversity and differentiation.

In this study, the average Heterozygoty and the percentage of polymorphic loci were slightly higher in the Kenyan medfly population ($H = 0.280$ & $P = 89\%$) compared to Moroccan medfly populations ($H = 0.254$ & $P = 81\%$), whereas the Shannon's index of the Kenyan medfly population was statistically different from the Moroccan one. It was estimated equal to 0.227 in the Kenyan population and varied between 0.175 for Tizi n'test and 0.122 for Aït Melloul. Using isozymes markers, Baruffi *et al.* (1995) and Malacrida *et al.* (1998) have found that the Heterozygoty of medfly populations were higher in the Kenyan samples (0.154 ± 0.043 & 0.161 ± 0.042) than the Moroccan ones (0.102 ± 0.037 & 0.097 ± 0.034). Nevertheless, their calculated standard errors were very high and the Heterozygoty of both populations might not be statistically different indeed. Recently, Kourti (2002) has analyzed the genetic diversity among medfly populations using isozymes and has estimated the Heterozygoty equal to 0.262 ± 0.008 in the Kenyan medfly population. This Heterozygoty of the Kenyan population is concordant with our data ($H = 0.261$) albeit using distinct markers, isozymes vs. RAPD, but Gasperi *et al.* (2002) reported that the Heterozygoty and the percentage of RAPD polymorphic loci of the medfly populations were considerably higher in the Kenyan sample ($H = 0.29 \pm 0.02$ & $P = 80\%$) than the Moroccan one ($H = 0.11 \pm 0.02$ & $P = 30\%$). These significant differences were not in total agreement with the calculated genetic parameters using other molecular markers and comparing diverse populations' data from Gasperi *et al.* (2002) and Malacrida *et al*. (2007). Our data agree to some extent with other laboratories' results, which show higher Heterozygoty and important percentage of polymorphic loci in the Kenyan medfly population compared to the Moroccan one and reveal a general decrease of genetic variation from supposed sources of medflies (Kenya) to invaded area (Gasparich *et al.*, 1997; Gomulski *et al.*, 1998; Malacrida *et al*., 1998; Gasperi *et al*., 2002). But our data show also comparable levels of polymorphism between Kenyan and some Moroccan populations, particularly in populations from higher altitudes. These discrepancies may be a consequence of sampling numbers and regions, which were considered at macrogeographical and microgeographical studies. It should be noted that previous macrogeographic studies have analyzed only one Moroccan sample, from the east coast of the Argan forest (probably at Aït Melloul near Agadir), whereas our study has taken into account many regions of the Argan forest. Also, it has shown additional DNA variability in the medfly populations from higher altitudes (Imariden, Tiz n'test, Kenya), compared to samples from lower altitudes (Aït Melloul, Essaouira & Amskroud). Hence, erroneous assumptions may be concluded from the macrogeographical studies if genetic diversity of differentiated medfly populations were not totally considered.

The F_{ST} reveals a very important genetic differentiation among medfly populations of the Moroccan endemic Argan forest ($F_{ST} = 0.448$), whereas the AMOVA analysis shows that significant amount of molecular variation (16.7%) is due to the differences between populations and reveals probably the medfly adaptation to geographic and climatic conditions. Moreover, our data indicate that the genetic structure of medfly populations is determined predominantly by altitude. Because the population geneticists were more interested in the latitudinal gradients than the altitudinal ones, only few papers reported the altitude effect on genetic variation in *Drosophila* (Sørensen *et al.*, 2005; Collinge *et al*., 2006). As the altitudinal variations occur relatively over small distances, the environmental conditions such as temperature, change more rapidly, compared with equivalent distances over latitudinal gradients. As a consequence, for a given temperature change, higher gene flow is more likely to happen along altitudinal gradients compared with latitudinal ones (Blanckenhorn, 1997). The environmental conditions, specifically the temperature may explain the genetic differentiation of Moroccan medfly population at different altitudes. In summer time, the temperature differences between the higher altitudes and the Atlantic coast are so important in the Argan forest and could reach 20°C difference (45°C to 25°C). The hot weather may result in higher mortality of medfly larvae of the Argan forests. As a consequence, the genetic variation may have increased

between medfly populations at different altitudes and altitudinal genetic differentiation can be more easily attributed to natural selection with temperature being a strong candidate selective agent.

The phyllogeographic clustering is very stable between the Kenyan and the Moroccan populations. The third internode of UPGMA dendrogram implies that the Moroccan medfly populations were polyphylitic, but it's not reliable since the Neighbor-Joining method reveals a single origin of Moroccan medflies. This discrepancy between the UPGMA and Neighbor-Joining dendrograms is a consequence of the Tiz n'test population branch and to a lower extent the Imariden one. Both of them were sampled at higher altitude near the Toubkal in the Higher ATLAS Mountains. Tiz n'test is one of the mountains links between the Sahara Desert and the fertile agricultural regions of Morocco. It may be the first region in Morocco, where the medfly has been established then invaded the other Moroccan agricultural regions. Thereafter, the trading North-South activity through the mountains has diminished during the last century and has moved mainly to Atlantic Ocean coast. Consequently the Tiz n'test region has been isolated. Indeed, The Tiz n'test medfly population shows the highest private bands, in comparison to other Moroccan medfly populations.

In conclusion, data clearly show occurrence of important genetic differentiation between Moroccan medfly populations and higher genetic diversity in medfly populations. Furthermore, the genetic structure of Moroccan medfly populations is determined predominantly by altitude.

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