Allozymic variability in Spanish populations of *Ceratitis capitata*

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Abstract — Introduction. The Tephritidae *Ceratitis capitata* is one of the most important agricultural pests in the world. Eradication programmes need as much genetic information as possible to be efficient. It is vital to know the genetic variation that exists in the areas thought to have been important in the expansion of the species, such as the Iberian Peninsula. The aim of this study was to determine the genetic structure of Spanish *C. capitata* populations and the relationships between them. **Materials and methods**. We studied the genetic variability of populations of *C. capitata* (eight wild samples and one laboratory strain) through the use of the horizontal starch gel electrophoresis technique and the assay of fifteen enzyme-coding loci chosen at random. **Results**. The quantity of variability detected in this polyphagous species was not high. Of the 15 loci studied, only four were clearly polymorphic. No significant differences were found in any comparison of populations collected from different hosts. **Discussion**. The distribution patterns of this variation seem to be the result of gene flow and selection in the form of agricultural practices.

Spain / *Ceratitis capitata* / electrophoresis / genetic variation

Variabilité parmi des allozymes présents dans des populations espagnoles de *Ceratitis capitata*.

Résumé — Introduction. *C. capitata*, Tephritidae, est l'un des principaux ravageurs des cultures. Pour l'éradiquer de façon efficace, il est nécessaire de disposer autant que faire se peut d'informations génétiques. Pour cela, il est essentiel de connaître la variation génétique existant dans les zones qui ont été importantes pour l'expansion de l'espèce, comme l'est la péninsule ibérienne. Le but de notre étude a été de déterminer la structure génétique de certaines populations de *C. capitata* espagnoles et les relations existant entre elles. **Matériel et méthodes**. Nous avons étudié la variabilité génétique de neuf populations de *C. capitata* (huit échantillons sauvages prélevés soit sur figues, soit sur pêches, et une souche de laboratoire) par électrophorèse horizontale sur gel d'amidon et analyse de quinze loci d'enzymes de codage choisis au hasard. **Résultats**. La variabilité détectée dans cette espèce polyphage a été faible. Parmi les 15 loci étudiés, seuls quatre se sont révélés clairement polymorphes. Aucune différence significative n'a été trouvée parmi les populations de *C. capitata* prélevés sur les différentes plantes hôtes. **Discussion**. Le type de distribution de cette variation semble le résultat de flux de gènes et de sélection imputables à des pratiques agricoles.

Espagne / *Ceratitis capitata* / électrophorèse / variation génétique

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1. Introduction

Ceratitis capitata (Wiedemann) is one of the most important agricultural pests in the world. Two main factors have made this so: the expansion of its host range from *Argaria spinosa* to more than 250 species and varieties of agriculturally important plants [1], and the ability to occupy new habitats worldwide. In less than two centuries, it has spread from its putative area of origin in Central Africa [2] to all countries with temperate or tropical climates, such as those of the Mediterranean, Central and South America, the Hawaiian Islands and Australia [3]. In addition, in recent years, several infestations have been detected on the mainland United States [4]. The fruit losses occasioned by this fly and the cost of prevention and eradication programmes make it an economically important pest in countries where it has become established or is re-introduced every year.

The number of genetic and molecular biology studies on insect pests has increased greatly in the last 15 years: advances in molecular technologies are helping to provide the genetic information very useful for making control programme decisions [5]. To be efficient, i.e., in order to eliminate pest populations or reduce them at sub-economic-injury levels, eradication programmes need information about the population structure, population history of the species, patterns of colonisation and monitoring the origin and spread of invading populations. PCR and DNA sequencing, etc., are now used to acquire this information since they require minute amounts of biological material, the markers are life-stage-independent, and most of them target non-coding regions, and hence are selectively neutral. Nonetheless, the groundwork with any species probably starts with gel electrophoresis (the oldest, simplest, one of the most cost-effective, and, until recently, the most widely-used technique [6, 7]). Gel electrophoresis provides a simple and unbiased method for quantifying variability, revealing mostly markers under selective pressure. The still-valid opinion of some authors is that there remains much to be gained from its use, both in the study of pests and in learning more about populations in general [7].

Several groups in Italy, Greece, the USA and our own group in Spain are trying to gain a better understanding of the genetic structure of *C. capitata* populations. Different aspects of the colonisation process, as well as the spatial and temporal relationships between populations have been extensively studied [4, 8–18]. However, much remains to be learned about the genetic variation between and within populations of that pest. It is vital to know the variation that exists in the areas thought to have been important in the expansion of the species. The Iberian Peninsula seems to have been the launching point for expansion to the Northern Mediterranean and perhaps to other regions. Unfortunately, data on Spanish populations are scarce [11, 15, 19, 20].

This paper reports the genetic variability (as determined by enzyme electrophoresis) of one laboratory and eight wild populations of *C. capitata* that were collected from different parts of Spain and from two kinds of hosts. The aim of our study was to determine the genetic structure of these populations and the relationships between them.

2. Materials and methods

2.1. Materials

Eight wild populations of *C. capitata* from different areas of Spain were studied (*figure 1*). The sites were chosen to cover the complete regional range of the medfly in Spain. Four of the natural populations were obtained from peaches, the other four from figs. Likewise, one laboratory population was analysed as an out-group. This strain was founded more than 30 years ago from an Eastern-Spanish population and is maintained at The Crops Protection Unit of the *Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica de Madrid* (ETSIA, UPM), Spain.

In all cases, adult flies were obtained by harvesting infested fruits and allowing the larvae to pupate under laboratory conditions (25 °C, 55% relative humidity). When adults emerged, they were frozen at –20 °C until the electrophoretic analysis. From each

Figure 1.
Sites of Spain where *Ceratitis capitata* populations were collected on figs or on peaches to study their allozymic variability.

population, some 100 to 300 flies were obtained from 30 to 50 fruits. Seventy to a hundred of these were randomly selected for analysis.

2.2. Electrophoresis techniques

Standard techniques for horizontal starch gel electrophoresis and the assay of enzymes were applied to individual adult flies, following the method of Ayala [21] with minor modifications. The same buffer system was always used: gel buffer 76 mM TRIS and 5 mM citric acid, pH = 8.65, electrode buffer = 300 mM boric acid and 60 mM NaOH, pH = 8.1 [21, 22].

Fifteen enzyme-coding loci were chosen at random for study: two loci for esterases (*Est-1*, *Est-3*), two for hexokinases (*Hk-1*, *Hk-3*), two for alcohol dehydrogenases (*Adh-1*, *Adh-2*) and one for aldehyde-oxidase (*Aox*), fumarase (*Fum*), fructokinase (*Fk*), α-glucophosphate dehydrogenase (α-*Gpdh*), hydroxybutarate-dehydrogenase (*Hbdh*), isocitrate dehydrogenase (*Idh*), malate dehydrogenase (*Mdh*), malic enzyme (*Me*) and phosphoglucomutase (*Pgm*).

A *Drosophila melanogaster* monomorphic strain (X_{9sod}) was used as a control in all gels. This strain was used as the reference "100" for allele designation in all cases.

2.3. Data analysis

Three classical parameters were employed for quantifying variability in the isozyme studies: P – polymorphism, the proportion of polymorphic loci (the 95% and 99% polymorphism criteria were both used), H – heterozygosity, i.e., the average number of heterozygous individuals, and n, the average number of alleles per locus. Departures from

the Hardy-Weinberg equilibrium were analysed by chi-squared analysis of heterozygotes. Nei's genetic distances [23] provided insights into the global divergence between every population pair, allowing the construction of a dendrogram (UPGMA method). Finally, Wright's method was used to determine the gene flow (derived from Wright's fixation indices [24]). The gene flow (*Nm*) was estimated from F_{ST} values using the relationship $F_{ST} = 1 / (4\,Nm + 1)$, i.e., $Nm = (1 - F_{ST}) / 4\,F_{ST}$, in which N is the effective population size and m the migrant proportion of the population.

3. Results

Of the 15 loci studied, only four were clearly polymorphic (*Aox, Est-3, Idh* and *Pgm*). One was completely monomorphic with the same allele fixed in all populations (locus *Hk-3*). The remainder were monomorphic in the majority of populations with the same fixed allele, although some rare alleles were seen at very low frequencies in some populations (loci *Adh-1, Adh-2, Est-1, Fk, Fum, Hbdh, Hk-1, Mdh,* and *Me*). The allelic frequencies at polymorphic loci, the proportion of heterozygous individuals (both those observed and expected under the conditions of the Hardy-Weinberg equilibrium), and the significance of their chi-squared analysis were assessed (*table I*). In most cases, the number of observed heterozygous individuals was significantly lower than that expected for the conditions of the Hardy-Weinberg equilibrium.

When analysing the three usual parameters for quantifying enzymatic genetic variation (polymorphism, heterozygosity and mean number of alleles per locus) (*table II*), variability seemed to be very similar for all nine populations. In fact, no significant differences were found in any Student *t* comparison of populations from different hosts (Student *t* values 0.32, –1.11, 0.68 and –0.11, respectively, $p > 0.30$ in all cases). The mean values change slightly if only the eight wild populations are taken into account.

Nm values were also calculated to investigate the possible influence of gene flow in the distribution of the variability found. The estimated mean value was 1.735 (using Wright's formula).

Nei's [23] genetic distances between all pairwise combinations of the nine studied populations were not very great (as expected), ranging between 0.0007 [between the Atajate and Rincón de Ademuz (2) populations] and 0.0605 (between the Mallorca and laboratory populations). The Mallorca population showed the greatest distances from all the other populations, except the Rincón de Ademuz (2) one. In general, the Rincón de Ademuz (2) population showed the lowest genetic distances from all the other populations. The dendrogram obtained with the method UPGMA shows two clusters, one with the island population of Mallorca, and the other with the remaining populations (*figure 2*). This second cluster displays two branches, one with the laboratory population on its own, the other containing the remaining populations.

4. Discussion

4.1. Degree of variability

Fifteen randomly-chosen loci – a random sample of the genome – were assayed. Singh and Rhomberg [25], who have compiled extensive variability and species data, conclude that doubling the number of studied loci has no significant effect on the proportion of polymorphic loci or on the mean heterozygosity. This, plus the fact that a large sample size was used (70 to 100 individuals in the majority of cases), suggests that the present data are representative of the true genetic variation of *C. capitata*.

The Mediterranean fruit fly is an extremely polyphagous species. According to some authors [26–28], a positive correlation can be expected between the genetic variation of a species and the degree of its environmental diversity. However, a comparison of the genetic variability of the populations studied here (representative of a wide subregional area) with those of other fruit flies (Diptera and Tephritidae) suggests that this may not be the case for the genetic variability of *C. capitata* (*tables I, II*). Its wide host

Table I.
Polymorphic loci: allelic frequencies, number of heterozygotes observed and expected according to the Hardy-Weinberg equilibrium, and chi-square results for nine populations of *C. capitata* collected on figs or peaches in different locations of Spain (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Loci and alleles	Laboratory population	Wild populations							
		Atajate	El Ejido	Madrid	Mallorca	Córdoba	Requena	Rincón de A. (1)	Rincón de A. (2)
Aox									
96	0.0093	0.0957	–	–	–	0.0556	0.0197	0.0435	–
98	0.9074	0.7766	0.8500	0.8334	0.2500	0.5833	0.8684	0.7391	1.0000
100	0.0833	0.1064	0.1167	0.1296	0.0909	0.3055	0.1118	0.1304	–
102	–	0.0213	0.0333	0.0370	0.6591	0.0556	–	0.0870	–
H. observed	4	3	4	3	5	7	8	16	–
H. expected	18,3***	17.7***	15.8***	7.8*	10.9*	10.1	17.7**	29.5**	–
Est-3									
98	–	–	0.0571	0.1333	–	–	0.0652	–	–
100	–	–	0.6000	0.4500	–	1.0000	0.3478	–	0.2206
102	1.0000	–	0.3143	0.4167	–	–	0.5870	–	0.7794
104	–	–	0.0286	–	–	–	–	–	–
H. observed	–	–	6	11	–	–	3	–	1
H. expected	–	–	14.6***	18.8**	–	–	12.2***	–	11.7***
Idh									
WS	0.0398	–	–	–	–	–	–	–	–
S	0.4829	0.5000	0.8055	0.7841	0.8108	–	0.6163	0.7381	0.5174
F	0.4773	0.5000	0.1945	0.2159	0.1892	–	0.3837	0.2619	0.4286
H. observed	63	3	26	17	0	–	62	22	–
H. expected	47.3***	13.5***	22.6	14.9	11.3***	–	40.7***	16.2	–
Pgm									
102	–	–	0.0085	–	–	–	–	–	–
106	0.0906	0.1122	0.0805	0.0625	0.1204	0.0426	0.1064	0.1193	0.1548
108	0.9094	0.8878	0.9068	0.9306	0.8796	0.9574	0.8936	0.6697	0.8452
110	–	–	0.0042	0.0069	–	–	–	–	–
112	–	–	–	–	–	–	–	0.2110	–
H. observed	27	20	21	10	5	4	8	28	13
H. expected	24.5	19.5	20.2	9.4	11.4*	3.8	8.9	530.7***	11.0

range does not translate into high values of heterozygosity, polymorphism or number of alleles per locus compared, for example, with the strict monophagous Tephritidae *Bactrocera oleae* [29] or other less polyphagous Tephritidae species such as *Rhagoletis pomonella* [30]. The question that arises is whether these low variation values are typical of the species or whether the populations studied are exceptional.

Previous studies of populations with different geographical origins have recorded even lower variability than that found here, with the exception of those in the area of origin [8, 10, 11, 31–33]. Similarly, previous investigations by our group on other populations show that, in general, Spanish populations are among the most variable of the species [14, 19, unpublished data].

Thus, even when only Spanish medfly samples have been analysed, the rate of genetic variability observed seems to be typical of the species. Variability seems to decrease as the distance from its origin increases, or the more recent the moment of colonisation. It is thought that the insect first invaded Spain (where it was detected in 1842 [1]) directly from Africa, then spread from the Iberian Peninsula to other northern Mediterranean countries and, finally, to the Middle East and other regions [2]. The present results (as well as previously published data

Table II.
Summary of variability observed in nine populations of *Ceratitis capitata* collected on figs or peaches in different locations of Spain.

Parameters studied	Laboratory population	Wild populations								Mean of the 9 populations	Mean of the 8 wild populations	Mean of the 4 fig populations	Mean of the 4 peach populations
		Atajate	El Ejido	Madrid	Mallorca	Córdoba	Requena	Rincón de A. (1)	Rincón de A. (2)				
Heterozygosity	0.09	0.03	0.06	0.08	0.03	0.05	0.10	0.09	0.04	0.06	0.06	0.05	0.07
Polymorphism 95%	0.27	0.25	0.29	0.31	0.27	0.18	0.33	0.27	0.30	0.26	0.25	0.27	0.24
Polymorphism 99%	0.38	0.54	0.43	0.38	0.27	0.31	0.46	0.50	0.38	0.41	0.41	0.41	0.41
Number of alleles per locus	1.73	1.69	2.00	1.61	1.33	1.55	1.67	1.82	1.40	1.64	1.63	1.66	1.61

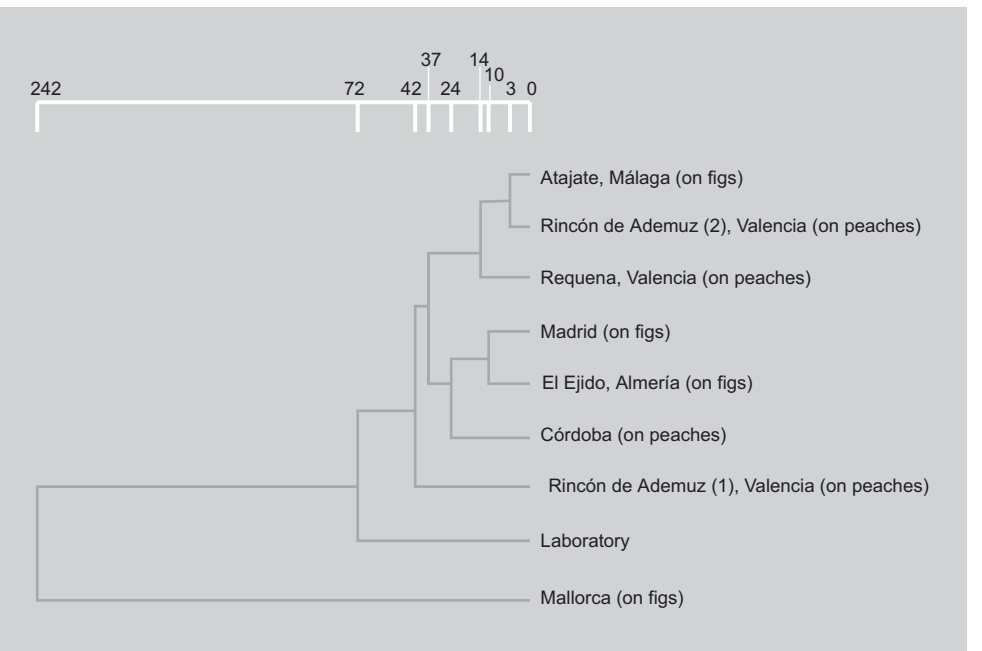

Figure 2.
Ceratitis capitata population relationships inferred from Nei's genetic distances measured between eight wild populations collected in different areas in Spain and a laboratory population (scale gives the Nei's genetic distances × 10^{-3}).

[14, 19, 20]) seem to support this hypothesis. As Malacrida *et al.* [10] indicate, "ancestral" populations, representative of the species' area of origin, are the most variable (sub-Saharan Africa), while "ancient" populations (those of the Mediterranean) show intermediate values of variability between these and "new" populations (*e.g.*, those of America). In summary, the proposed hypothesis on the possible correlation between the degree of environmental diversity and degree of genetic variability does not hold in this species.

Another possible explanation of this low variation is the adaptation of the medfly to different fruit hosts. *C. capitata* feeds on many different types of fruits, depending on availability. Consecutive generations, depending on the time of the year, must be adapted to different host fruits. This would tend to restrict variability since natural selection

would pick more versatile "generalist" alleles that could serve (though not necessarily perfectly) for different host fruits [20]. In this respect, no differences between populations from different hosts (figs and peaches) were detected (*tables I*, *II*). When data for populations collected on figs (Atajate, El Ejido, Madrid and Mallorca) are compared with those of populations collected on peaches [Córdoba, Requena and Rincón de Ademuz (1) and (2)], the results are virtually the same. There are no significant differences between any comparable figures ($p > 0.30$ for Student t values, in all cases).

The genetic variability of the laboratory population was similar to that of the wild populations. Two things probably account for this: the time elapsed since the population was established (more than 30 years) and the provision of "fresh" flies. Both events may have helped a positive adaptation to laboratory conditions. This would allow the population to support a certain degree of variability. No significant differences in the quantity of variability were detected in comparisons between laboratory and wild populations for any parameters.

4.2. Patterns of variability: intra- and inter-population variation

For the present data, the first characteristic of note in the analysis of intra-population variability is the lack of agreement with Hardy-Weinberg expectations (*table I*). This is so for all four variable loci analysed and for the majority of the populations (17 out of 28 tests). In all cases except two (*Idh* in the laboratory and Requena populations), this was due to a deficiency of heterozygotes. Although other authors [29, 34] have observed this same tendency with other species of Diptera, this does not appear to be a general situation.

The heterozygous deficiency detected in the present medfly populations could be due to different causes, such as the presence of null alleles, the Wahlund effect, or inbreeding. However, given that no null homozygotes were detected, that samples were obtained from uniform fields, that it is highly unlikely that the eight different populations came from different sub-populations, and that captures were carried out between August and October after several generations of expansion, these possibilities are very improbable.

The explanation must be related to selection pressures or "family structure". The sampling of "families" seems possible in *C. capitata* because females of this species tend to lay several eggs on each fruit [3]. However, sampling was undertaken at a time of high fruit availability, and several dozen fruits were collected in every case, from which large numbers of randomly-selected flies were assayed. Selection is therefore probably working on these populations, at least on some of the loci.

With respect to the inter-population distribution of the variability detected, the basic characteristic observed was that, for all loci (except *Aox* in the Mallorca population and *Est-3* in three populations), the same allele is either fixed or has the highest frequency. Further, the allele with the second highest frequency was also the same (except in the same two cases). A random drift cannot explain this similarity. Drift effects should be uncorrelated in the different populations.

Gene flow, with its tendency to make populations uniform, cannot be disregarded. Therefore, attempts were made to infer the role played by gene flow in relation to the genetic structure of the populations. In the case of *C. capitata*, data on migratory capacity have shown the medfly to be capable of dispersing over great distances, even though low vagility is normal when there are abundant fruit trees [3]. Wright's method [24] showed the mean gene flow between our populations to be $Nm = 1.735$. This seems to indicate that significant, although not excessive, gene flow exists among the populations studied. Human-mediated movements probably play an important role in gene flow among these populations. Fruit-growing areas, such as the southeast and east of Spain, provide these foods to the rest of the country, thus the El Ejido and Rincón de Ademuz (1) and (2) areas could easily provide flies to Córdoba and Madrid. Thus, gene flow would definitely seem to play some role in the distribution of variability of

the studied populations, especially in its geographic uniformity.

Only two populations were clearly differentiated from the others, Mallorca and laboratory. But these are special populations, one from an island and the other a laboratory strain. Thus, both are to some degree isolated populations, and it seems logical that they might be different. With respect to the other seven populations, however, there were no qualitative differences, and only a few quantitative differences in loci variables were seen (*table I*).

In agreement with the deviation from the Hardy-Weinberg equilibrium, it seems probable that gene flow occurs at a relatively significant rate, at least among seven of the populations [Atajate, El Ejido, Madrid, Córdoba, Requena and Rincón de Ademuz (1), and (2)]. That local variation in selection intensity (perhaps caused by differences in insecticide use and pest control measures, in general) is strong enough to maintain low differentiation among populations.

The phylogenetic tree resulting from the Nei's distances is very illustrative (*figure 2*). Three clear groups stand out: one represented by Mallorca, another by the laboratory strain and a third with the rest. This latter group shows no clear tendency, either with respect to the climate or the geography of the sampled areas. This would appear to add weight to the idea that natural selection in the form of different agricultural practices (different crops, cultivated areas, method and time of pesticide use, etc.) is responsible, at least in part, for the variability seen.

In summary, three important results emerge from our present study. First, the amount of variability detected in these populations of *C. capitata* is relatively low compared with that observed in other Tephritidae species, disagreeing with the expected relationship between variability and the degree of environmental diversity. Second, gene flow could be significant given the "general" similarity in the most frequent alleles. Third, it is reasonable to conclude that selection in the form of agricultural practices, in combination with gene flow, is responsible for the geographic patterns of allozyme variation observed. Efforts to fight this pest must take into account the level of gene flow between different geographic areas and there must be greater coordination in agricultural practices from different administrations.

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Variabilidad aloenzimática en poblaciones españolas de *Ceratitis capitata.*

Resumen — Introducción. *Ceratitis capitata*, Tephritidae, constituye una de las plagas agrícolas más importantes del mundo. Es necesario disponer de una mayor información genética sobre esta especie con el fin de desarrollar programas de erradicación más eficaces. Para ello, resulta fundamental conocer la variación genética existente en las zonas que han jugado un papel importante en la expansión de dicha especie, como la Península Ibérica. El objetivo del presente estudio ha sido determinar la estructura genética de las poblaciones españolas de *C. capitata* y las relaciones existentes entre ellas. **Material y métodos**. Hemos estudiado la variabilidad genética de nueve poblaciones españolas de *C. capitata* (ocho poblaciones naturales y una de laboratorio) mediante la técnica de electroforesis horizontal en gel de almidón y el análisis de quince loci enzimáticos elegidos al azar. **Resultados**. Los niveles de variabilidad genética encontrados en esta polífaga especie no han sido elevados. De los quince loci estudiados, sólo cuatro fueron claramente polimórficos. No se han observado diferencias significativas entre poblaciones procedentes de diferentes frutos hospedadores. **Discusión**. Los patrones de distribución de la variabilidad genética parecen ser el resultado de la acción del flujo génico y de la selección, bajo la forma de prácticas agrícolas.

España / *Ceratitis capitata* / electroforesis / variación genética