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Adh$_1$ first intron polymorphisms in Argentinean populations of *Ceratitis capitata*

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Abstract

DNA size polymorphisms were utilized in a study of 24 natural populations of *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) from Argentina. The first intron of alcohol dehydrogenase 1 gene (Adh_1) was amplified using exon priming intron crossing-polymerase chain reaction. Three size variants were detected among the 307 samples analyzed. To better differentiate the size variants, further digestion of PCR products with the *Eco*RI restriction enzyme was carried out. Complete nucleotide sequences of the three-allele variants were obtained and single changes, insertions, deletions, and *Eco*RI recognition sites were located. Population allele frequencies were analyzed and a global mean heterozygosity (He) of 0.33 was obtained. In most populations, observed allelic frequencies conformed to Hardy–Weinberg expectations. Significant differences between provinces and sampling sites within these provinces, and among some populations were found. The average number of insects exchanged among populations (Nm) was estimated and high values were observed between Argentina and populations from two African countries (Morocco and Kenya), Australia, and Hawaii (Kauai). Pest introduction sources and dispersion patterns in Argentina are discussed based on these results as well as on available bibliographical data.

Introduction

Ceratitis capitata Wiedemann (Diptera: Tephritidae), commonly known as the medfly, is one of the most important fruit and vegetable pests with an extensive variety of host species (Liquido et al., 1991). It has been able to adapt to a variety of environmental conditions and has migrated from its origin in equatorial Africa to tropical and temperate regions around the world (Carey, 1991). Its presence in Argentina was first recorded in an orchard near the city of Buenos Aires (Vergani, 1952). It was later reported in the commercial orchards of the north-eastern and north-western regions of the country (cf. Segura et al., 2006). It causes significant economic losses in infested areas, mainly because of direct damage to the fruit and the restrictions imposed on fresh fruit imports to 'medfly-free' countries. The PROCEM (Programa Nacional de Control y Erradicación de Moscas de los Frutos) has obtained medfly-free areas in Argentina (Cosenzo, 2003; Malavasi & Mangan, 2004), mainly by means of toxic baits and Sterile Insect Technique (SIT; Knipling, 1959).

Molecular markers are powerful tools in eradication programs against agricultural pest species. In particular, the alcohol dehydragenase (*Adh*) system has potential utility in genetic sexing for the improvement of SIT (Robinson et al., 1986), and in germ-line transformation (Robinson et al., 1988), and it provides useful markers for population analysis. The *C. capitata Adh* system consists of two tightly linked genes Adh_1 and Adh_2 resulting from a gene duplication on the left arm of chromosome 2 (Malacrida et al., 1992). Both genes exhibit different temporal and developmental expression profiles (Gasperi et al., 1992, 1994). The coding regions of both genes are interrupted by introns. In general, *Adh* introns are relatively short

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(between 70 and 162 bp) for insects, with the exception of the first intron of Adh_1 in *C. capitata*, which is very long and polymorphic in size.

Nuclear introns with unknown sequences can be analyzed by exon priming intron crossing-polymerase chain reaction (EPIC-PCR) using complementary primers to flanking exons with conserved sequences across species (Lessa & Applebaum, 1993; Slade et al., 1993). There are several examples of EPIC-PCR analysis of population structure and bioinvasion dynamics of invasive insect populations (Palumbi & Baker, 1994; He & Haymer, 1997; Villablanca et al., 1998). Moreover, Gomulski et al. (1998) studied the size variation of the Adh_1 first intron in 16 populations of *C. capitata* from five geographical regions around the world. These authors demonstrated an association between the geographical dispersal of the medfly from its source area and a gradual reduction in intron variability. A similar loss in variability was deduced using allozyme analysis (Malacrida et al., 1998). The nucleotide sequence of Adh_1 first intron in *C. capitata* was determined and further phylogenetic analysis suggested that the shortest intron variant (Ke-1400) and a medium size variant (Ke-1970) may represent the ancestral forms of the intron, whereas the longest variant evolved later (Gomulski et al., 2004).

Here, we studied the variability of the first Adh_1 intron in 24 wild populations of *C. capitata* from Argentina using EPIC-PCR. Analyses of population structure and variability distribution were performed to understand the genetic aspects of the colonization of South America, as well as to obtain information on the evolutionary potential of this pest.

Materials and methods

Population sampling and DNA extraction

Ceratitis capitata adult flies and larvae were collected between 1998 and 2002. Adult flies were sampled using Jackson or McPhail traps from the provinces of Mendoza, Río Negro, San Juan, and La Rioja. Larvae found inside infested fruit were collected from the remaining provinces and reared to adulthood under laboratory conditions. The insects (either trapped or reared from fruit) were washed with TE buffer (10 mM Tris–HCl, 10 mM EDTA, pH 8) and stored at -20 °C until DNA isolation. Total DNA was isolated from adult flies based on the protocol described by Baruffi et al. (1995).

In total, 307 individuals were analyzed from 17 provinces (24 sampling sites), representing the main fruit-producing regions of Argentina. The sampling sites were grouped into six biogeographical regions (Cabrera & Willink, 1980): 'Las Yungas' [La Rioja B (LRB), La Rioja C (LRC), Salta (SA), Jujuy P (JUP), Jujuy Y (JUY), Tucumán 1 (TU1), Tucumán 2 (TU2), Tucumán 3 (TU3)], 'Chaqueña' [Córdoba (CD), Santa Fe (SF), Corrientes 2 (CO2), Santiago del Estero (SG), Formosa (FS), Catamarca (CA), Chaco (CH)], 'Pampeana' [Entre Ríos 1 (ER1), Entre Rios 2 (ER2), Buenos Aires (BA)], 'Monte' [San Juan (SJ), Mendoza 1 (ME1), Mendoza 2 (ME2), Río Negro (RN)], 'Espinal' [Corrientes 1 (CO1)], and 'Paranaense' [Misiones (MI)] (Table 2).

Polymerase chain reaction amplification

The Adh_1 first intron was amplified using EPIC-PCR and NESTED-PCR (using the PCR product as a template). Amplification products were separated by electrophoresis in 1.5% (wt/vol) agarose gel in 0.5× TBE buffer (Sambrook et al., 1989), with a 1 kb DNA ladder (Invitrogen, Carlsbad, CA, USA) as a molecular weight marker.

Exon priming intron crossing-polymerase chain reaction reactions were carried out in 20 µl final volume in buffer (50 mM Tris–HCl pH 8.3; 2% sucrose; 3 mM $MgCl_2$; 250 µg ml^{-1} BSA; 200 µM dNTPs; 1 ng $µl^{-1}$ DNA; 0.5 µM primers; *Taq* polymerase 1 U per assay) using ADH1 (5′-GGAAGCTTGCTTATGAAGCCTGCA-AGT-3′) and ADH2 (5′-ACTTTGGTTTTTGGGTTGAT-TTCC-3′) primers described by Gomulski et al. (1998). The amplification program has an initial denaturalization step of 2 min at 94 °C, 35 repeating cycles of 30 s at 94 °C, 1 min at 63 °C, and 3 min at 72 °C, followed by a final temperature ramp of 0.1 °C s^{-1} from 72 to 50 °C in a Mastercycler Gradient Eppendorf Thermocycler (Eppendorf, Hamburg, Germany).

NESTED-PCR was performed using EPIC-PCR product as a template (3 µl of 1:50 dilution) with ADHfn (5′-ATGAACAATGACTTGGC-3′) and ADHrn (5′-CGT-CGAACACGAACAACG-3′) primers, designed in this study based on the available DNA sequence (AN Z30194) of the flanking exon regions. They are located 27 bases internal to the original forward primer, and 49 bases internal to the original reverse primer. The cycling program includes a 2-min denaturation step at 94 °C, 40 repeating cycles of 30 s at 94 °C, 30 s at 55 °C, and 2 min at 72 °C in a Mastercycler Gradient Eppendorf Thermocycler.

NESTED-polymerase chain reaction product digestion

Representative NESTED-PCR fragments of the detected size variants were analyzed with 14 restriction enzymes: *Eco*RI, *Hind*III, *Cla*I, *Bam*HI, *Pst*I, *Eco*RV, *Vsp*I, *Eco*ICRI, *Xma*I, *Hpa*II, *Eco*52I, and *Bst*EII (Promega, Madison, WI, USA), and *Mnl*I and *Mse*I (New England Biolabs, Ispwich, MA, USA). Polymerase chain reaction product (200 ng) was incubated for 2 h at 37 °C with 1 U of each restriction enzyme (separated assays) in the appropriate reaction

buffer. The restriction products were visualized by electrophoresis in 2% (wt/vol) agarose gel in 0.5× TBE buffer (Sambrook et al., 1989).

Size-variant inheritance

To study size-variant segregation in the offspring, 14 pairs of adult individuals (parents) and their brood were analyzed. Adult flies were reared and mated under laboratory conditions in IGEAF INTA, Castelar, Buenos Aires, Argentina. DNA samples from parents and offspring were analyzed by EPIC-PCR, NESTED-PCR, and *Eco*RI (5′ G▼AATTC 3′) (Promega) restriction assay. Three parent couples were selected according to their size-variant distribution. A minimum of 10 individuals from their brood were randomly selected and analyzed.

Sequencing of the Adh_1 first intron

The PCR products of three size variants of the Adh_1 first intron detected were eluted from agarose plugs and cloned using pGEM-T Easy kit (Promega). Sequences were obtained using an automatic ABI PRISM™ 377 Sequencer (Applied Biosystems, Foster City, CA, USA) with a 'DYEnamic ET terminator cycle sequencing kit' (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Data analysis was performed in a Macintosh computer system using SEQUENCING ANALYSIS ver. 3.4.1 and SEQED ver. 1.0.3 programs (Applied Biosystem). Intron walking was initiated with vector primers. Afterwards, the following primers were used (in this order): ADH1, ADH2 (Gomulski et al., 1998), ADH-SF1 (5′-GAGGTCAAAGCGAATTCACC-3′), ADH-SR1 (5′-CGAAGGGTTATCCATC-3′), R1 (5′-GAATTGCCGAACATGATGGC-3′), F2 (5′-AGCAAGCTCAGAAGGCCG-3′), F1 (5′-GGAGTTAGTCAAGTTGTGACG-3′), 4R3 (5′-GCGGTATTTCTACAATAAATC-3′), 4R2 (5′-ACATAAGATCGGCCTTTCGC-3′), and 3R2 (5′-CGAATTGACAGCGGCAGTCTCG-3′). These primers were designed from the sequences obtained.

The sequences were processed using Staden Package (Staden, 1996) and aligned using Bioedit (Hall, 1999) and ClustalX 1.81 (Thompson et al., 1997). Consensus sequences of each allele were compared with the nucleotide sequence databases, using the US National Institute of Health's Blastn 2.2.10 (Basic Local Aligment Search Tool; Altschul et al., 1990).

Population variability analysis

Allele frequencies, heterozygosity (Nei, 1987), and Hardy–Weinberg equilibrium (Guo & Thompson, 1992) were estimated for each sampling site using Arlequin v. 2.000 (Schneider et al., 2000). The Kruskal–Wallis test was employed to compare H estimates among populations using the STATISTICA program (StatSoft, 2001).

Samples of provinces were classified into biogeographical regions to analyze genetic structure. Sampling sites within each province were grouped in future analysis, except for CO1 and CO2 which are located in different biogeographical provinces. The distribution of genetic variability at various hierarchical levels was analyzed using two approaches, viz. analysis of F-statistics (Wright, 1951) and analysis of molecular variance (AMOVA) (Excoffier et al., 1992). F-statistics were estimated using the BIOSYS program (Swofford & Selander, 1981) and AMOVA was performed using the Arlequin v. 2.000 software (Schneider et al., 2000).

Pairwise differentiation analyses of provinces and sampling sites were performed using the Fisher exact test with TFPGA program (Miller, 1997). Pairwise gene flow (Nm: numbers of migrants per generation) was estimated using the method described by Slatkin (1985), which is based on the distribution of rare alleles. The Nm pairwise comparison with other regions of the world was performed using allele frequencies estimated by Gomulski et al. (1998) and the frequencies estimated in the present work from the sampling performed in Argentina.

Results

Only three size variants of approximately 2 700, 2 600, and 2 000 bp, were identified in 307 individuals from wild populations of *C. capitata* in Argentina using EPIC-PCR (Figure 1A). A NESTED-PCR was developed to increase sensitivity and specificity. The size variants were scored as 2 515, 2 461, and 1 952 according to the NESTED-PCR product size determined by sequencing. A screening of restriction enzymes was performed so that the three alleles could be scored. Fourteen restriction enzymes were included. Digestion with *Eco*RI facilitated the differentiation between similar-sized variants (2 515 and 2 461), which were separated using agarose electrophoresis (Figure 1B).

Size-variant segregation analysis

Three parent couples and 10 individuals from their offspring were genotyped for the Adh_1 first intron. The segregation obtained indicated a simple Mendelian segregation of the three alleles at the Adh_1 locus (Table 1).

Sequence analysis

Complete sequences of the three alleles were obtained. The sequences exhibited high variability, including point mutations and insertion/deletions (indels). The most important indel (562 bp coordinates 1 444–2 006; Figure 2) explained the size differences between variant 1 952 and the two other sequences, whereas the presence of

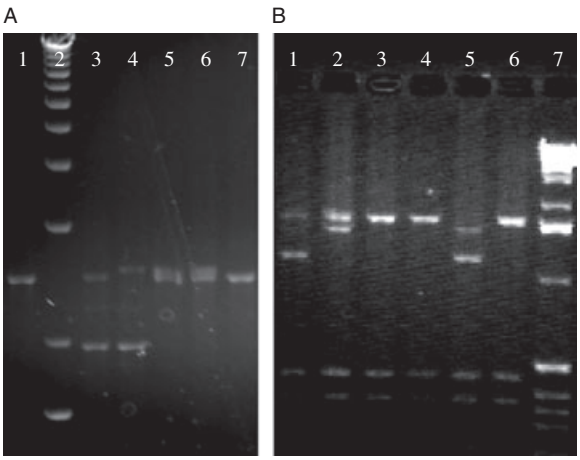

Figure 1 Adh_1 first intron size variants of *Ceratitis capitata*. (A) Lanes 1, 3–7: EPIC-PCR products of San Juan individuals with genotypes 2 461/2 461, 2 461/1 952, 2 515/1 952, 2 515/2 461, 2 515/2 461, and 2 461/2 461, respectively. Lane 2: 1 Kb DNA ladder (Invitrogen, Carlsbad, CA, USA). (B) Size variants *Eco*RI digestion. Lanes 1–6: NESTED-PCR products of six individuals from San Juan with genotypes 2 461/1 952, 2 515/2 461, 2 461/2 461, 2 461/2 461, 2 515/1 952, and 2 461/2 461, respectively. Lane 7: 1 Kb DNA Ladder.

shorter indels explained the differences between size variants 2 515 and 2 461.

Consensus sequences from two clones of each variant were compared with the NCBI nucleotide database. Size variant 1 952 (GQ503646) showed a 99% similarity with the sequence AY 426620 (variant Be-2060 described by Gomulski et al., 2004). A 96% similarity was observed between size variant 2 461 (GQ503645), and the published sequence AY426619 (described as variant Be-2590 by Gomulski et al., 2004). There was no identical sequence to size variant 2 515 (GQ503644), although lower similarities were detected with some entries. We suggest that 2 515 corresponds to variant 2 670 described by Gomulski et al. (1998) and we provide its nucleotide sequence here.

Table 1 Adh_1 first intron size-variant segregation in the offspring from controled crosses in *Ceratitis capitata*. Parents and offspring genotypes were scored according to the size variants obtained by NESTED-PCR as 2 515, 2 461, and 1 952

	Offspring (no. individuals)			
Cross	2 461/ 2 461	2 461/ 2 515	2 461/ 1 952	1 952/ 1 952
2 461/1 952 × 2 461/2 461	5		5	
2 461/2 461 × 2 461/2 515	4	5		
1 952/1 952 × 2 461/2 461			10	

Mariner pseudo-sequences (coordinate 364–1 010 in Figure 2) with 641 bp were identified in the nucleotide sequences of all three alleles. The ITR (inverted terminal repeats) sequence found at the extreme of this pseudo-sequence showed identical nucleotide sequence to the ITR of *Ccmar*1 transposed element (Gomulski et al., 1997; GenBank accession number U76905).

The introns of variants 2 515, 2 461, and 1 952 are 2 479, 2 425, and 1 917 bp long, respectively, and the size of the EPIC-PCR products are 2 606, 2 551, and 2 043, respectively. The structure and size of the Adh_1 variants were analyzed and *Eco*RI restriction sites were located on the nucleotide sequence (Figure 2). Allele 2 515 shows three *Eco*RI restriction sites, whereas alleles 2 461 and 1 952 include only two sites for this restriction enzyme. *Eco*R1 digestion of variant 2 515 generates four DNA fragments of 341, 459, 1 545, and 170 bp. Three fragments upon *Eco*R1 digestion were obtained for 2 461 and 1 952 variants, with sizes of 336, 455, and 1 670 bp for variant 2 461, and 339, 454, and 1 159 bp for variant 1 952.

Population variability: Adh_1 first intron analysis

Of the Adh_1 first intron variants detected, allele 2 461 is widespread in all populations studied, whereas alleles 2 515 and 1 952 were found in much lower frequency (Table 2). A high level of genetic variation was found in *C. capitata* from Argentina. All sampling sites studied, except JUP, were polymorphic for the Adh_1 first intron. The expected mean heterozygosity (He) was 0.33 and the mean observed heterozygosity (Ho) was 0.28. The observed and expected heterozygosity within the sampling sites showed a considerable amount of variation. The observed heterozygosity ranged from 0 to 0.71 and expected heterozygosity ranged from 0 to 0.59 (Table 2). Kruskal–Wallis tests indicated that biogeographical region did not have a significant impact on the genetic diversity measurement ($P>0.05$). Three (CO2, MI, and SF) out of the 24 sampling sites exhibit deviations from the expected values of Hardy–Weinberg equilibrium (Table 2).

Hierarchical analysis of variation through AMOVA (Table 3) did not reveal significant differences between biogeographical regions, but significant differences ($P<0.05$) were detected between and within provinces (Table 3). Similar results were obtained when the F_{ST} hierarchical analysis was performed (Table 4). Both analyses showed that most variations detected could be explained by a significant differentiation among provinces, whereas no significant differentiation was observed between biogeographical regions in any analysis.

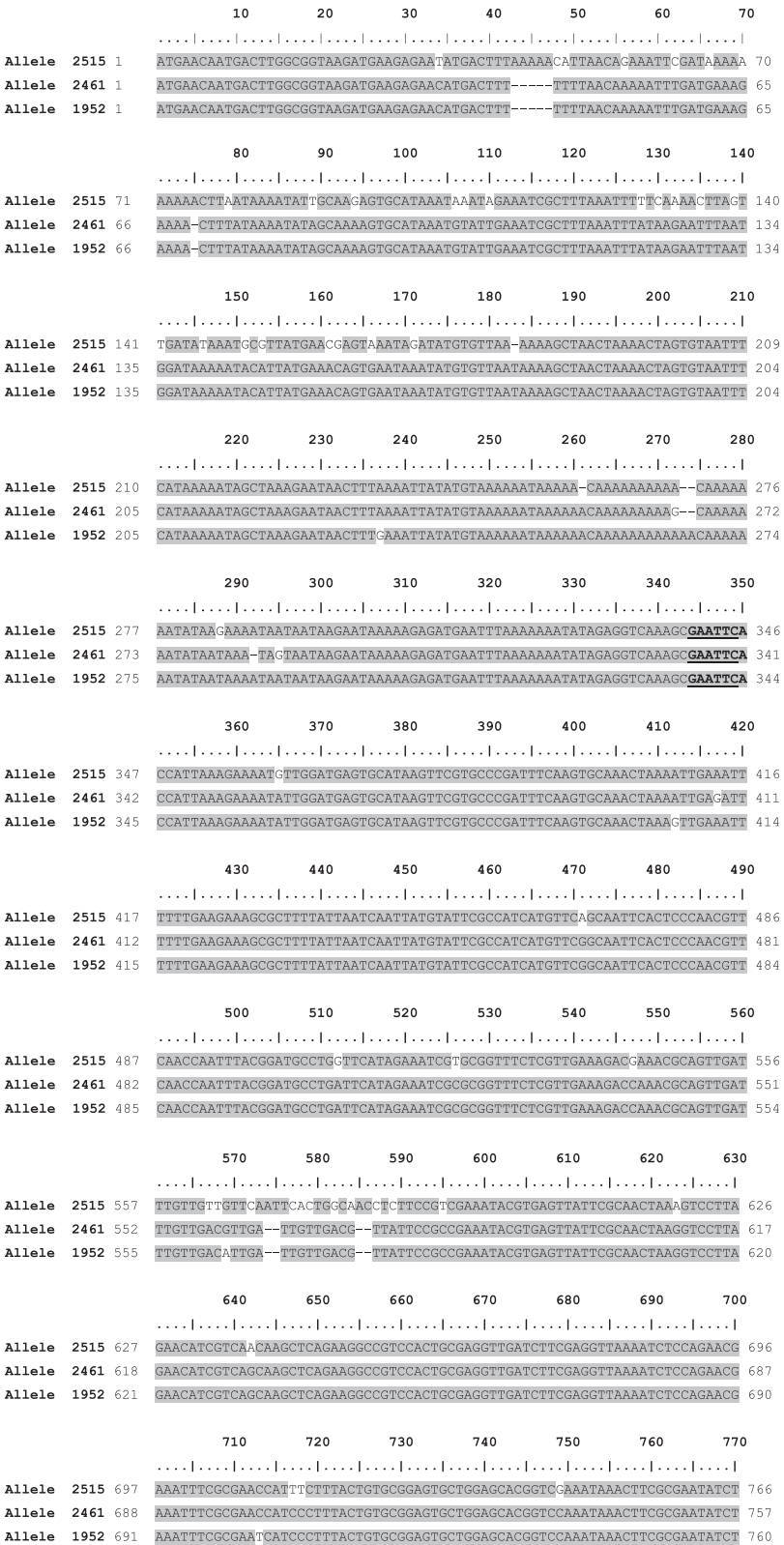

Figure 2 *Adh$_1$* first intron sequence alignment in *Ceratitis capitata* Argentinean populations. *Eco*RI recognition sites are in bold and underlined, nucleotide identities are in gray.

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                        780       790       800       810       820       830       840
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Allele  2515 767 TTAGATGAAGCGCCTACTGTTTTTCCTTTGCGGAATTCGTATTTGTAACATAATATGACGCATATGATCT 836
Allele  2461 758 TTAGTTGCAGCGCCTACTGTTTTTCCTTTGCGGAATTCGTAT----AACATAATTTGACGCATATGATCT 823
Allele  1952 761 T-AGTTGCAGCGCCTACTGTTTTTCCTTTGCGGAATTCGTAT----AACATAATTTGGCGCATATGATCT 825

                        850       860       870       880       890       900       910
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Allele  2515 837 TTTTCGTTGTCCATCTTCCATGCGTCTTTTCACTAAGCAACTCAAATCAAAATGAAGTTATTTTAAGCTT 906
Allele  2461 824 TTTTCGTTGTCCATCTTCCATGCGTCTTTTCACTAAGCAACTCAAATCAAAATGAAGTTACTTTAAGCTT 893
Allele  1952 826 TTTTCGTTGTCCATCCAAATCAAAATAAAGTTATTTTTGCTTGATTTACAGTTAGG--ACTGTCACCTT 893

                        920       930       940       950       960       970       980
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Allele  2515 907 GATTTATACGGTATATATATTTCTTTCACGAGCGCAACTCTCAAACGATTCGAACGGCATCCAGCGGAAT 976
Allele  2461 894 GATTTATATGGTATATATATTTCTTTCACGAGCGCCACTCTCAAACGATTCGAACGCCATCCAGCGAAAT 963
Allele  1952 893 ---TCCAACG--ATATATATTTCTTTCACGAGCGCCACTCTCAAACGATTCGAACGGCATCCAGCGGAAT 958

                        990       1000      1010      1020      1030      1040      1050
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Allele  2515 977 CGGGCACGAACTTATGCACTCATCCAATATAAACCAAGAAAAATATATCAAATGATTTATTTAAAAAAAA 1046
Allele  2461 964 CGGGCACGAACTTATGCACTCATCCAATATAAACCAAGAAAAATATATCAAATGATTTATTTAAAAAAAA 1033
Allele  1952 959 CGGGCACGAACTTATGCACTCATCCAATATAAACCAAGAAAAATATATCAAATGATTAATTTAAAAAAAA 1028

                        1060      1070      1080      1090      1100      1110      1120
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Allele  2515 1047 GTTGAATTTCAATTCTTCTTCAGAAACTGGAGCATGAATGAAAATATAAAATTAAAACAAATAAATGAGT 1116
Allele  2461 1034 GTTGAATTTCAATTCTTCTTCAGAAACTGGAGCATGAATGAAAATATAAAATTAAAACAAATAAATGAGT 1103
Allele  1952 1028 -TTGAATTTCAATTCTTCTTCAGAAACTGGAGCATGAATGAAATTATAAAATTAAAACAAATAAAAGAGT 1097

                        1130      1140      1150      1160      1170      1180      1190
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Allele  2515 1117 AAATTTTCTTTTTCTTAATTAA---AAAAAATTTAACTCAAAACAAATCAACACCTGCTTTATACAAATA 1183
Allele  2461 1104 AAATTTTCTTATTCAAAATTAACAAAAAAAATTAAACGCAAAACAAATCAACGCCTGCTTTATACAAATA 1173
Allele  1952 1098 AAATTTTCTTTTTCTTAATTAA---AAAAAATTAAGCTCAAAACAAATCAACGCCTGCTTTATACAAATA 1164

                        1200      1210      1220      1230      1240      1250      1260
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Allele  2515 1184 CTGTTTTTT-CAAAATATTTTAAATTCAATTCTCAATAAAGGGGTCCTAGTTTTCAAACTTTATAATAAA 1252
Allele  2461 1174 CTGTTTTTT-CAGAATATTTTAAATTCAGTTCTCAATAAAGGGGTCTTAGTTTTCAAACTTTTAATAAA 1242
Allele  1952 1165 CTGTTTTTTTCAAAATATTTTAAATTCAATTCTCAATAAAGGGGTCCTAGTTTTCAAACTTTATAATAAA 1234

                        1270      1280      1290      1300      1310      1320      1330
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Allele  2515 1253 AAAATGTCGACAAAATAATTTAATTTAAAAGAAAATATAGAAAATTTAAAGCAACTTATATATGCCGTAA 1322
Allele  2461 1243 AAAATGTCGAAAAAAAAATGTCA------AAAAAGATAGAAAAATTATAGGAACTTATAAATGCCGTAA 1306
Allele  1952 1235 AAAGTGTCGACAAAATAATTTAATTTAAAAGAAAAGATAGAAAATTTAAAGCAACTTATATATGCCGTAA 1304

                        1340      1350      1360      1370      1380      1390      1400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Allele  2515 1323 TTTTATGATTTATTGTAGAAATACCGCAAACAAACTAATATTTGAAAATTATTAGATTTTTTAATTTTAA 1392
Allele  2461 1307 TTTAATGATTTATTGTAGAAATACCGCAAACAAACTAATATTTGAAAAATATTGGATTTTTTGATTTTAA 1376
Allele  1952 1305 TTTTATGATTTATTGTAGAAATACCGCAAACAAACTAATATTTGAAAATTATTAGATTTTTTAATTTTAA 1374

                        1410      1420      1430      1440      1450      1460      1470
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Allele  2515 1393 AACTTTATATACAACAATACAAATTCAATAATAATACAATTGAAAATATACTTAATTTAGTTTAGAATA 1462
Allele  2461 1377 AACTTCATATATAGCAATACACAATACATTAATAATACAATTGAAAATATACTTAATTTAGTTTAGAATG 1446
Allele  1952 1375 AACTTTATATACAACAATACACAATACAATAATAATACAATTG---------------------------- 1417

                        1480      1490      1500      1510      1520      1530      1540
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Allele  2515 1463 AATTTACATATCAAAATTACGTGATCTCCAATGTACCGAGCTGAATTTTGAAAATTATCAAACTTAATTA 1532
Allele  2461 1447 AATTTACATATCAAAATTATGTGATTTCCAATGTACTGGGCTGAATTTTGAAAATTATCAAACTTAATTA 1516
Allele  1952 1417 ------------------------------------------------------------------- 1417
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Figure 2 Continued

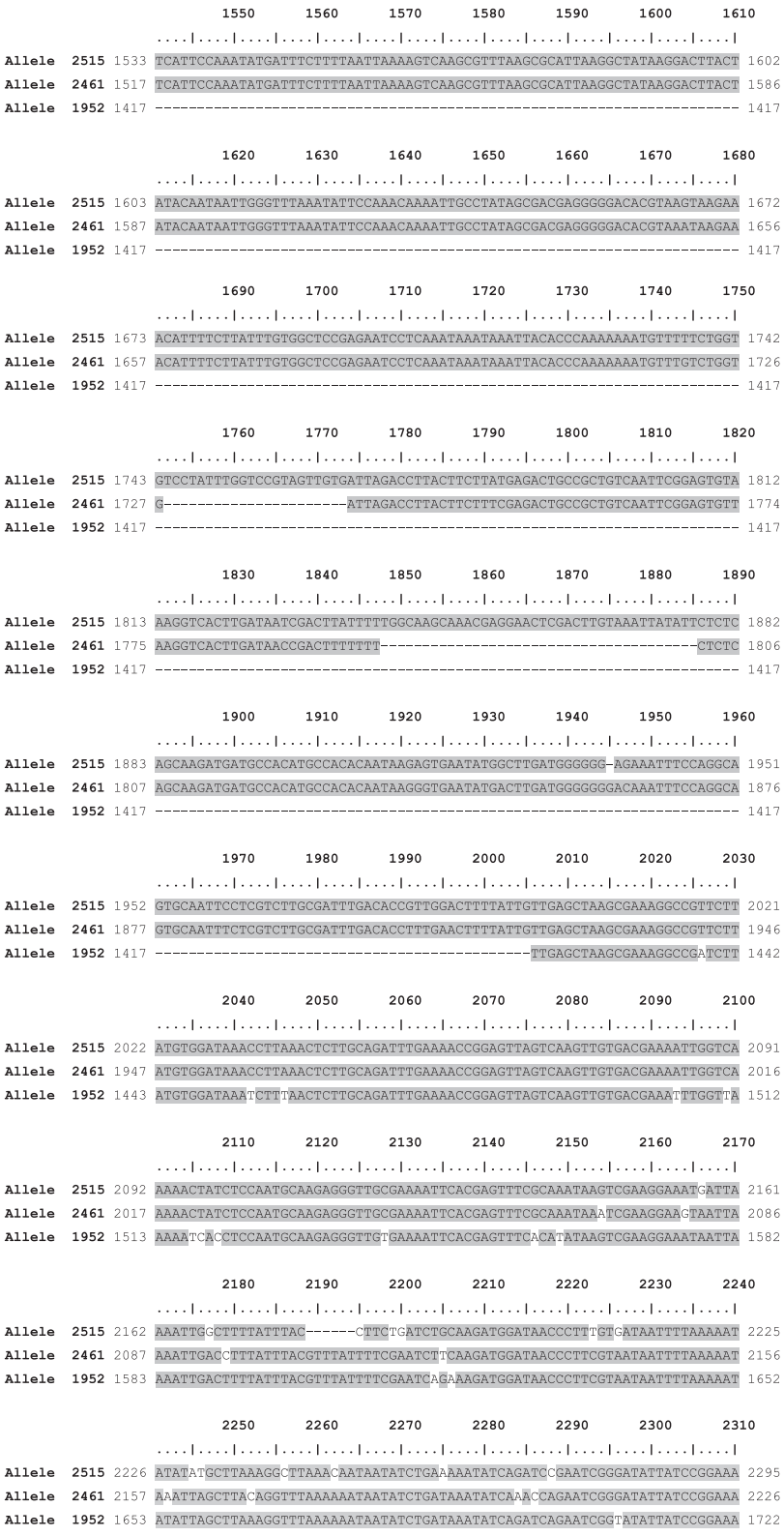

Figure 2 Continued

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                    2320      2330      2340      2350      2360      2370      2380
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Allele  2515 2296 AAATTAAAATTTGTTAATAGAAAT---ATGTTTACCAAAATATGTTGTAGAGAATTCAAAACAAATACAC 2362
Allele  2461 2227 AAATTAACAATAATCAAAACAAATTATATGTTTACGAAAATATGTTGTAAAGAATTTAAAAAAAATACAC 2296
Allele  1952 1723 AAATTAACACTAATTAATATAAAT---ATGTTTAGCAAAATATGTTGCAAAGAATTAAAAAAAAATACAC 1789

                    2390      2400      2410      2420      2430      2440      2450
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Allele  2515 2363 TTTTTATAAAATAAATTTTTTTTTTTTGAAAGAAAAGCTATCCCCTAAAATTGTTGTATTGCAAAATTAT 2432
Allele  2461 2297 TTTTTATAAAATAAATTTTTTTTTTTTGAAAGAAAAGCTATCCCCTAAAATTGTTGTATTGCTAAATTAT 2366
Allele  1952 1790 ATTTTATAAAATAA--TTTTTTTTTTTGAAGGAAAAGCTATCCCCTAAAATTGTTGTATTGCAAAATTAT 1857

                    2460      2470      2480      2490      2500      2510      2520
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Allele  2515 2433 TACCCTCTAATGATATTGCATTTA----------TTTCTATCAATTACAAAAAACAATCTCTTCTCTT 2490
Allele  2461 2367 TACCCTCTAATAATATTGCATTTATTTCTATTTTTATTTCTATCAATTACAAAAAACAATCTCTTCTCTT 2436
Allele  1952 1858 TACCCTCTAATAATATTGCATTTATTTATATTTTTATTTCTATCAATTACAAAAAACAATCTCTTCTCTT 1927

                    2530      2540
                    ....|....|....|....|.
Allele  2515 2491 CTTCAGTCGTTGTTCGTGTTCGACG 2515
Allele  2461 2437 CTTCAGTCGTTGTTCGTGTTCGACG 2461
Allele  1952 1928 CTTCAGTCGTTGTTCGTGTTCGACG 1952
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Figure 2 Continued

Table 2 Allele frequencies and genetic diversity parameters for the *Adh*$_1$ first intron in 24 populations of *Ceratitis capitata*

Biogeographical region	Province	Sampling site	n	Allele frequency			Heterozygosity		P-value
				2 515	2 461	1 952	Ho	He	
Las Yungas	Jujuy (JU)	JUP	7	0.000	1.000	0.000	0.000	0.000	–
		JUY	6	0.250	0.667	0.083	0.330	0.530	0.510
	La Rioja (LR)	LRB	15	0.000	0.867	0.133	0.270	0.240	1.000
		LRC	15	0.067	0.867	0.067	0.270	0.250	1.000
	Salta (SA)	SA	15	0.200	0.767	0.033	0.330	0.39	0.590
	Tucuman (TU)	TU1	7	0.143	0.857	0.000	0.290	0.260	1.000
		TU2	7	0.071	0.857	0.071	0.290	0.280	1.000
		TU3	14	0.071	0.929	0.000	0.140	0.140	1.000
Chaqueña	Catamarca (CA)	CA	15	0.167	0.733	0.100	0.400	0.440	0.130
	Cordoba (CD)	CD	14	0.071	0.929	0.000	0.140	0.140	1.000
	Chaco (CH)	CH	15	0.200	0.800	0.000	0.130	0.330	0.050
	Formosa (FS)	FS	8	0.125	0.875	0.000	0.250	0.230	1.000
	Santa Fe (SF)	SF	13	0.500	0.423	0.077	0.150	0.590	<0.001**
	Santiago del Estero (SG)	SG	8	0.000	0.813	0.188	0.380	0.330	1.000
	Corrientes (CO)	CO2	7	0.286	0.643	0.071	0.140	0.540	0.020*
Espinal		CO1	7	0.357	0.643	0.000	0.710	0.490	0.440
Monte	Mendoza (ME)	ME1	15	0.233	0.733	0.033	0.400	0.420	1.000
		ME2	15	0.200	0.667	0.133	0.530	0.520	0.520
	Rio Negro (RN)	RN	15	0.033	0.933	0.033	0.130	0.130	1.000
	San Juan	SJ	15	0.100	0.867	0.033	0.200	0.250	0.200
Pampeana	Buenos Aires	BA	15	0.100	0.800	0.100	0.400	0.350	1.000
	Entre Rios (ER)	ER1	15	0.167	0.800	0.033	0.330	0.340	0.190
		ER2	29	0.155	0.793	0.052	0.410	0.350	1.000
Paranaense	Misiones	MI	15	0.133	0.833	0.033	0.130	0.300	0.020*
Mean value				0.151	0.796	0.053	0.281	0.327	
SE							0.030	0.030	

*Significant differences; **highly significant differences (Guo and Thompson test).
n, number of analyzed individuals; Ho, observed heterozygosity; He, expected heterozygosity.

Table 3 Analysis of molecular variance (AMOVA) among biogeographical regions and provinces of *Ceratitis capitata*

Source	Variance	Total (%)	F
Among biogeographical regions	−0.00410Va	−2.24	F_{CT} = −0.02238
Among provinces within biogeographical regions	0.01722Vb	9.41	F_{SC} = 0.09201*
Within provinces	0.16990Vc	92.83	F_{ST} = 0.07169*
Total	0.18302	100	

*P<0.05. F = F-statistics: Va, Vb, and Vc represent the molecular variation among biogeographical regions, provinces within biogeographical regions, and within provinces, respectively.

Table 4 F-statistics hierarchical analysis among biogeographical regions, provinces, and provinces within regions in *Ceratitis capitata*

Comparison	Variance	F_{XY}	% genetic diversity
Among biogeographical regions (CT)	−0.00888	−0.013	−1.3
Among provinces (ST)	0.03645	0.052	5.2
Among provinces within a region (SC)	0.04533	0.064	6.5

F-statistics (F_{xy}) are indicated for each level of hierarchical population structure (Wright, 1978).

The Fisher exact test for population differentiation revealed significant and marginally significant genetic differences between provinces in the Chaqueña (P = 0.0031) and Las Yungas (P = 0.082) biogeographical regions (Figure 3; Table 5).

The 'pairwise differentiation test' for province and sampling site comparisons shows the amount of significant differentiation between provinces (Figure 3A) and sampling sites (Figure 3B) in relation to each population; SF, ER, and SG provinces were the most differentiated, and FS, JU, and SA provinces were the least differentiated. Sampling sites SF, CO1, and LRB were the most differentiated, whereas TU2, JUY, and SA were the least differentiated. Differentiation within provinces was not observed in ER, JU, LR, ME, and TU, but significant differentiation was found between CO sites (CO1 and CO2). In addition, sampling site CO2 was different from its neighboring ER sites (ER1 and ER2) (Table 5).

The average number of insects exchanged among worldwide populations (Nm) was estimated. Comparisons were performed between the Argentinean population (mean frequencies) and other populations reported by Gomulski et al. (1998). High and moderate values of Nm were observed between Argentina and both African populations, Morocco (54.71) and Kenya (5.60). Significant numbers of migrants per generation were also found between Argentina and the Australian (4.19) and Hawaiian (Kauai 3.89) populations. The comparison with populations of the Mediterranean region showed a relative low Nm value (1.93).

Discussion

Ceratitis capitata is a colonizing species that has spread from its putative source area in Africa to many countries in four main geographic regions: the Mediterranean basin, South and Central America, Australia, and the Pacific basin. Colonization processes have been analyzed using several molecular markers (Gasperi et al., 1991; Malacrida et al., 1992, 1996, 1998, 2007; Gasparich et al., 1995; Gomulski et al., 1998; Lanzavecchia et al., 2008). Some authors suggest that medfly populations could be divided according to the colonization patterns in three main groups: ancestral, ancient, and New World populations (Malacrida et al., 1998).

Variations in nuclear introns have previously been used to analyze medfly population structure and colonizing processes (Gomulski et al., 1998, 2004; Villablanca et al.,

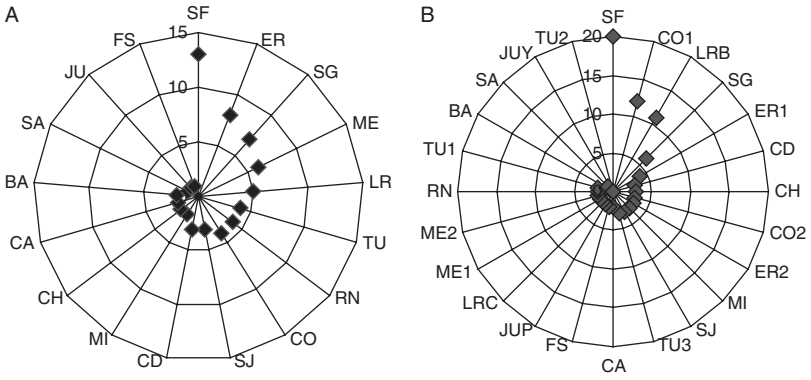

Figure 3 Fisher pairwise differentiation test among (A) 17 provinces and (B) 24 sampling sites of *Ceratitis capitata*. The radial axis shows the numbers of populations (provinces or sampling sites) with significant differentiation (P<0.05) in relation to each population.

Table 5 Fisher pairwise differentiation test between sampling sites of *Ceratitis capitata* from Argentina

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
(1) BA																							
(2) CA	-																						
(3) CD	-	-																					
(4) CH	-	-	-																				
(5) CO1	-	-	+	+																			
(6) CO2	-	-	-	-	+																		
(7) ER1	-	-	-	-	-	+																	
(8) ER2	-	-	-	-	-	+	-																
(9) FS	-	-	-	-	-	-	-	-															
(10) JUP	-	-	-	-	+	-	-	-	-														
(11) JUY	-	-	-	-	-	-	-	-	-	-													
(12) LRB	-	+	-	+	+	-	+	+	+	-	-												
(13) LRC	-	-	-	-	+	-	-	-	-	-	-	-											
(14) ME1	-	-	-	-	-	-	-	-	-	-	-	+	-										
(15) ME2	-	-	-	-	-	-	-	-	-	-	-	+	-	-									
(16) MI	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-								
(17) RN	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-							
(18) SA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
(19) SF	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+					
(20) SG	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	+				
(21) SJ	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-			
(22) TU1	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-		
(23) TU2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
(24) TU3	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-

(+) Significant differences ($P<0.05$) between sampling sites.

1998; Davies et al., 1999). The Adh_1 first intron varies extensively in medfly populations around the world with 18 reported size variants (Gomulski et al., 1998). Studying 307 insects from 24 wild populations of Argentina, we found only three Adh_1 size variants (2 515, 2 461, and 1 952) in agreement with the three variants described for Argentina by Gomulski et al. (1998).

At the sequence level, we found high similarities between the two smaller size variants (2 461 and 1 952) and the Be-2590 and Be-2060 variants described by Gomulski et al. (2004). The longest variant characterized here (2 515) has previously been described. We suggest that 2 515 corresponds to the variant 2 670 described by Gomulski et al. (1998).

When the relative allele frequencies of the three variants detected were analyzed, we found size variant 2 461 widely distributed at very high frequency (80%) in Argentina, similar to the allele frecuency found for variant 2 590 in populations around the world (Gomulski et al., 1998). This variant was also present in the putative African area of origin and in all colonized populations. Size variants 2 515 and 1 952 were detected at low frequencies (15 and 5%, respectively) within the populations studied here. These values are similar to the allele frequencies of the 2 670 and 2 060 variants found for Argentina by Gomulski et al. (1998). Size variant 2 060 has previously been detected in Africa (0.03–0.47%) and in almost all colonized areas (except Australia), whereas size variant 2 590 was found in Africa (0.06–0.23%), Pacific populations (0.15–0.35%), and Australia (0.06–0.14%), but has not been detected in the Mediterranean basin (Gomulski et al., 1998). Differentiation of these size variants in colonization areas may be the result of genetic drift and/or bottlenecks. However, as suggested by Gomulski et al. (1998), we cannot exclude the possibility that during the colonization process some kind of selection was involved maintaining a subset of the ancestral intron variants.

Recently established populations generally present less variability than populations found in the geographic area where the species originated. Using the Adh_1 first intron as molecular marker, high levels of genetic variability have been detected in ancestral African populations (He = 0.61–0.93), whereas lower estimated values of genetic diversity have been found in ancient populations (old derived populations) from the Mediterranean basin and Australia (He = 0.09–0.30 and 0.11–0.25, respectively).

Argentinean populations exhibit moderate values of genetic diversity (He = 0.33), suggesting a more complex colonization process in agreement with studies with mitochondrial markers (Lanzavecchia et al., 2008).

Most of the populations (sampling sites) studied here exhibited Hardy–Weinberg equilibrium. Only 3 out of the 24 sampling sites analyzed exhibited deviations from the expected values. In these cases, an excess of homozygous individuals suggests the existence of a population sub-structure. Natural populations of *C. capitata* do not occupy a uniform environment. Rather, ephemeral and heterogeneous patches (the fruit) are used as breeding and feeding sites by small number of individuals (Civetta et al., 1990). The grouping of individuals in a delimited area (sampling site) could lead to population stratification.

The AMOVA showed that pest distribution is not associated with a biogeographical pattern and detected a significant differentiation between provinces and within populations. The absence of genetic differentiation at the biogeographical level might be explained by the high genetic differentiation between provinces within each biogeographical region, suggesting stochastic processes molding genetic variation of each population.

Analysis of allelic frequencies through pairwise comparisons revealed that populations from Santa Fe were the most differentiated. Significant differentiation was detected between both Entre Ríos sampling sites (ER1 and ER2) and the CO2 site. Additionally, CO1 and CO2, two sampling sites from the same province (Corrientes), are genetically differentiated. These results suggest that environmental, bioecological, and commercial factors may contribute to differential distribution of allele frequencies in neighbor sampling sites. The sampling site in the province of Buenos Aires did not show any significant differences with other sampling sites, probably because of the fact that Buenos Aires is the center of the commercial route in Argentina. The most important trade routes of fruit and vegetables are BA-ME, BA-ER, ME-TU, and CO-TU (ISCAMEN, 1997, 2000, 2001-2002). According to our results and previous ones (Bertin et al., 2007; Malacrida et al., 2007), we can conclude that transport and other human activities are very important in the dispersion of pests and other insect species both within Argentina and throughout the world.

The genetic variability distribution analyzed here was compared with mitochondrial haplotype variation (PCR-RFLP) obtained for the same populations from Argentina (Lanzavecchia et al., 2008). To this end, we re-analyzed the mitochondrial data at the biogeographical level. The analysis of molecular variance did not reveal significant differences between biogeographical regions (F_{CT} = 0.77, P = 0.23), whereas significant differences were detected among provinces and sampling sites (F_{SC} = 6.69, P<0.0001: F_{ST} = 92.54, P<0.0001, respectively).

Genetic differentiation patterns obtained using mitochondrial and nuclear markers showed similar genetic differentiation between provinces and within populations. However, the pairwise differentiation test for mtDNA (data not shown) rendered 76 out of 136 significant differences, whereas according to the data presented here (Table 5), 46 out of 276 pairwise comparisons rendered significant differences for the Adh_1 first intron. In some insect species, analysis of nuclear and mtDNA genes revealed different variation patterns (Kim & Sappington, 2004; Sesarini & Remis, 2008). The higher level of genetic differentiation among pairs of sampling sites detected with a maternally inherited marker may be partially explained by the high rate of evolutionary change of the mtDNA or by sex-biased disersal.

The average number of migrants exchanged between colonized areas in Argentina and putative ancestral and older derived areas (described by Gomulski et al., 1998) revealed that Argentinean populations of *C. capitata* maintain relatively high levels of gene flow with Kenyan and Moroccan populations, suggesting that there are infestation sources for the Argentinean populations. Moreover, these populations showed high Nm values with populations of Australia and Hawaii. The relatively low genetic differentiation between Argentinean population and these putative source areas can be attributed to high gene flow per se or to shared ancestral polymorphisms. Because Argentinean populations were recently established, the second hypothesis might be more likely. The high levels of gene flow between Argentina and Kenya and Morocco found by Gomulski et al. (1998) are related to the critical trade routes to and from the Mediterranean basin and the Americas and Pacific area. Additionally, Malacrida et al. (2007) indicate that more recent and rapid pest expansion to Latin America and the Pacific appears to be a consequence of the opening of new trade routes and increasing human mobility. The lack of information regarding the distribution of Adh_1-intron1 alleles in neighboring countries prevents further inferences on pest dispersal patterns in Latin America.

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