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Recent changes in the distribution of carboxylesterase genes and associated chromosomal rearrangements in Greek populations of the tobacco aphid *Myzus persicae nicotianae*

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We present data on the frequency of amplified *E4* and *FE4* carboxylesterase genes in *Myzus persicae s.l.* clones collected during the years 2002–2007 and 2012 in Greece. Most clones were of the tobacco aphid, *Myzus persicae nicotianae*. Samples from 2012 were genotyped with microsatellite DNA markers and a number of them were karyotyped. Aphid clones with amplified *FE4* genes predominated in all years, whereas *E4* was present in only 3.5% of all samples and always occurred in clones with *FE4*. Most of the clones examined showed high carboxylesterase activity levels (R2 resistant category). The results showed marked changes in the frequencies of the two carboxylesterase genes in the tobacco aphid populations compared to published data that were collected in Greece in the mid 1990s, when *E4* was recorded on its own in 20% of all samples and in 32% of samples from tobacco. A parallel change in karyotype was also observed because the A1,3 translocation, which had a worldwide association with amplified *E4* genes in the 1990s, was not detected in the clones analyzed in 2012. Possible causes for these changes are discussed, although selection as a result of pest management practices appears to be the major one. Novel chromosomal rearrangements were also found in *M. persicae nicotianae* clones. These rearrangements could be a result of clastogenic effects of nicotine, which could persist because of the holocentric nature of aphid chromosomes. The results are discussed in relation to rapid evolution events that have taken place in the tobacco aphid in Greece during the last two decades.

ADDITIONAL KEYWORDS: insecticides – karyotype – microsatellite DNA – selection.

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INTRODUCTION

Aphids (Hemiptera: Aphidoidea) are an ideal study model for both applied and theoretical biologists. A unique combination of traits, such as alternation of sexual and asexual generations, genetically and environmentally determined phenotypes (polyphenism), and rapid population growth, provides aphids with the required genetic variation and ecological flexibility to succeed in diverse environmental conditions and ecosystems (Loxdale & Lushai, 2007). Aphids are also important agricultural pests, and the most important group of plant-virus vectors, causing severe crop losses. Extensive chemical control programmes are applied against them, leading to the rapid evolution of insecticide resistance through various resistance mechanisms, often associated with a fitness cost for the aphids (Foster, Devine & Devonshire, 2007). Insecticide resistance influences the genetic properties and structure of aphid populations under pressure (Zamoum *et al*., 2005). Many studies on aphid pests have used microsatellite DNA markers along with insecticide resistance markers to track the movement/dispersal of resistant genotypes in various agroecosystems and to determine the factors promoting their ecological success and the development of resistance (Fenton *et al*., 2005; Brévault *et al*., 2008). Some studies have focused on temporal dynamics[e.g. in *Myzus persicae* (Sulzer) (Hemiptera: Aphididae)] and reported large changes in the frequencies of resistant phenotypes through the years in England (Foster *et al*., 2002) or clonal turnover of resistant genotypes in Scotland (Kasprowicz *et al*., 2008). These changes were attributed to selection not only related to insecticide application, but also to other traits of the aphids' bioecology (host-plant, winter severity, fitness cost associated with resistance) and to the dispersal/introduction of new aphid genotypes.

In the 1990s, intensive studies were made of Greek populations of *M. persicae s.l.* These included work on insecticide resistance mechanisms (Blackman *et al*., 1999; Cox, Denholm & Devonshire, 2004), life-cycle variation and host preferences (Zitoudi *et al*., 2001; Margaritopoulos *et al*., 2002), morphological variation (Margaritopoulos *et al*., 2000), and microsatellite DNA genotyping (Blackman *et al*., 2007; Margaritopoulos *et al*., 2007a). The results established that the tobacco-adapted subspecies *Myzus persicae nicotianae* Blackman (known as tobacco-aphid) predominated everywhere in Greece that tobacco *Nicotiana tabacum* L. (Solanaceae) was grown not only on tobacco, but also on other crops and weeds. In north central Greece, its host plants include peach *Prunus persica* L. (Rosaceae), on which sexual reproduction occurs in autumn and overwintering eggs are laid. Subsequent work has revealed that isolating mechanisms have developed to restrict interbreeding on peach between *nicotianae* and *persicae s.s.* (Margaritopoulos *et al*., 2007a). Microsatellite and multivariate morphometric analyses of *persicae s.l.* populations on a global scale indicate that the host shift to tobacco occurred only once, probably in eastern Asia (Blackman, 1987; Margaritopoulos *et al*., 2007b; 2009). In addition, the genetic changes that enable *nicotianae* to detoxify nicotine have now been identified (Bass *et al*., 2013). Various studies (Blackman *et al*., 2007; Margaritopoulos *et al*., 2007a; Zepeda-Paulo *et al*., 2010) have shown that *nicotianae* in Greece exhibits high levels of genetic variation compared to other countries. This is attributed to the ability of the tobacco aphid to reproduce sexually in Greece. The agricultural practices and especially the chemical control against the aphid exert strong selection pressure and thus provide the opportunity to examine evolution on a sufficiently short timescale such that changes can be monitored.

Myzus persicae commonly resists insecticides by the overproduction of carboxylesterases, involving the amplification of two genes, *E4* and *FE4* (Devonshire & Field, 1991; Blackman *et al*., 1995, 1996; Field *et al*., 1999). Before 2000, *E4* was closely linked to a particular autosomal A1,3 translocation (i.e. a partial reciprocal translocation between the first and third autosome pairs, observed as heterozygous in karyotypes with a standard chromosome number $2n = 12$), whereas amplified *FE4* sequences occurred at several chromosomal locations in aphids of normal karyotype ($2n = 12$). Whether aphids had the *E4* or the *FE4* amplified genes also had a strong connection with life-cycle category, clones with *E4* and the translocation usually had all-year-round parthenogenesis and occurred commonly in warmer parts of the world or in glasshouses, or in places where peaches were not grown (such as central and southern Greece). By contrast, *FE4*-based resistance was most common where there was a regular sexual phase on peach (such as in northern Greece). This was probably because translocation carriers would be at a disadvantage in the sexual phase because a proportion of their gametes would be inviable as a result of genetic imbalance (and translocation homozygotes are also inviable) (Blackman *et al*., 1995, 1996, 1999).

This pre-2000 association between carboxylesterase gene, karyotype, and life-cycle category applied not only in Greece, but also in other parts of the world. It also applied to both *persicae s.s.* and *nicotianae*, which both have the same amplified carboxylesterase genes (Field *et al*., 1994). Presumably, in each case, these amplified sequences were acquired by the tobacco aphid in rare hybridization events, followed by the

intensive selection of the new highly advantageous combination of insecticide resistance and the ability to colonize tobacco.

However, in the late 1990s, the first evidence was obtained of a change in the frequency and distribution of the two carboxylesterase genes in Greek populations, with all clones examined carrying the *FE4* gene (Denholm & Cox, 2002). It became apparent that other changes were occurring, although it was unclear whether they were in a transient phase or fixed. The long-term persistence of these genotypes and the underlying evolutionary causes (e.g. deterministic or stochastic events) have not been examined nor clarified. Thus, to monitor these changes, we sampled populations in the years 2002–2007 for the occurrence of *E4* and *FE4*, and in 2012 for a more detailed study of microsatellite genotypes, resistance levels, and karyotypic variation.

MATERIAL AND METHODS

APHIDS

Myzus persicae samples were collected from northern (NG) (years 2002–2007 and 2012), central (CG) (years 2003–2006 and 2012), east-central (ECG) (years 2003–2007), and southern (SG) Greece (years 2004–2007) from peach orchards, tobacco and pepper *Capsicum annum* L. (Solanaceae) fields, and surrounding weeds. Most of the samples (78.4%) were from tobacco-growing localities. Peach trees and weeds in non tobacco-growing areas were also surveyed (peach in ECG and in one locality in CG and SG; weeds in ECG) (Table 1, Supporting Information Fig. S1). On the basis of previous findings (Margaritopoulos *et al*., 2000, 2005, 2007a; 2009), a reasonable working assumption is that the samples collected in the tobacco-growing regions of Greece are *nicotianae*. Peach orchards were surveyed in April and May (before aphid migration),

Table 1. Hosts and regions surveyed in the present study (sampling years in brackets) and type of genetic analyses that clones from each sample were subjected

Region	Host	Locality	Esterase activity	PCR-based diagnostics	TaqMan†	Microsattelite DNA markers
North Greece (NG)	Peach	Meliki (2003–2007)	+	+		
		Meliki (2012)	+		+	+
		Katerini (2006)	+	+		
		Velvendos (2006–2007)	+	+		
	Tobacco	Meliki, (2002–2007)	+	+		
		Meliki (2012)	+	+		
		Katerini (2004–2007)	+	+		
		Kria Vrisi (2004)	+	+		
		Velvendos (2006–2007)	+	+		
		Milia (2012)	+		+	+
	Pepper	Meliki (2012)	+		+	+
	Weeds	Meliki (2006–2007)	+	+		
		Meliki (2012)	+		+	+
Central Greece (CG)	Peach	Tirnavos* (2012)	+		+	+
	Tobacco	Karditsa (2003–2005)	+	+		
	Weeds	Karditsa (2006)	+	+		
Eastern-Central Greece (ECG)	Peach	Lehonia* (2003–2007)	+	+		
		Velestino* (2003–2007)	+	+		
	Weeds	Lehonia* (2006)	+	+		
		Velestino* (2006)	+	+		
South Greece (SG)	Peach	Argos* (2006–2007)	+	+		
	Tobacco	Amfiklia (2004–2005)	+	+		
		Prosymni (2004–2005)	+	+		
	Weeds	Amfiklia (2006)	+	+		
		Prosymni (2006)	+	+		

The life-cycle category was examined in most of the clones and a number of the 2012 clones was karyotyped.
*Non tobacco-growing localities.
†A number of the 2012 clones was also examined with polymerase chain reaction (PCR)-based diagnostics. For details, see Material and methods.

and herbaceous crops from June to August. Aphids from weeds were usually collected during winter, except the 2012 samples, which were collected in spring and summer. One wingless parthenogenetic female from each aphid sample was used to establish a parthenogenetic lineage (clone) in the laboratory. Clones were reared on excised leaves of Chinese cabbage *Brassica rapa pekinensis* Hanelt (Brassicaceae) in aphid rearing boxes (Blackman, 1971) at 17 °C and under an LD 16 : 8 h photocycle. Specimens from each clone were stored in 95% ethanol at –20 °C for DNA analyses.

The life-cycle category of almost all aphid samples was examined, by rearing clones for three consecutive generations under short-day conditions (SD; LD 10 : 14 h) at 17 °C. The morph of the first 60–80 individuals produced in the second generation under SD was identified upon reaching adulthood. This test allows the discrimination of holocyclic (cyclical parthenogens, producing sexual females and males), anholocyclic (obligate parthenogens) and intermediate (functional parthenogens, producing a few males and sexual females) genotypes. However, it does not guarantee the distinction between androcyclic (functional parthenogens, producing a few males but no sexual females) and anholocyclic genotypes, for which the whole sequence of progeny from many females is required (Margaritopoulos *et al*., 2002).

Microsatellite DNA genotyping of the 2012 samples

Aphid clones from 2012 were genotyped with microsatellite DNA markers. Genomic DNA was extracted from individual aphids with the 'sodium hydroxide' method (Malloch *et al*., 2006). Six microsatellite loci (M35, M40, M49, M63, M86, and myz9), which have been isolated from *M. persicae* (Sloane *et al*., 2001), were used for the genotyping. Microsatellite loci amplification, analysis and visualization are described in a previous study (Malloch *et al*., 2006). The different multilocus genotypes (MLGs) among the aphid parthenogenetic lineages and the probability that individuals sharing the same MLG are products of different sexual reproductive events (P_{SEX} statistic) were estimated using MLGsim, version 2.0 (Stenberg, Lundmark & Saura, 2003; http://www.rug.nl/fmns-research/theobio/downloads). The clonal diversity index P_d (= G/N) was calculated and for the subsequent analyses only one copy of each MLG per sample was used. This is a common method for aphids because clonal 'amplification' is not equal over all genotypes (Sunnucks *et al*., 1997). Mean number of alleles per locus (N_a), proportion of null alleles [A_N; *sensu* Brookfield, (1996)], observed (H_O) heterozygosity, expected (H_E) heterozygosity, and inbreeding coefficient (F_{IS}) over all loci were calculated using GENEPOP, version 4.1.3 (Rousset, 2008; see also http://genepop.curtin.edu.au). Deviations from Hardy–Weinberg equilibrium (HWE) at each locus and across all loci were examined using the score test (*U*-test) and the multi-sample version and linkage disequilibrium between pairs of loci using the *G* log-likelihood based exact test as implemented in GENEPOP. Allelic richness (R_s = number of alleles independent of sample size) was calculated using FSTAT, version 2.9.3.2 (Goudet, 1995; http://www2.unil.ch/popgen/softwares/fstat.htm).

Total carboxylestarases activity

An immunoassay was initially developed to quantity the amount of the *E4*/*FE4* carboxylesterases and categorize the *M. persicae* individuals in four arbitrary defined categories (S, R1, R2, and R3 = susceptible, moderately, highly, and very highly resistant, respectively; Devonshire, Moores & ffrench-Constant, 1986; Foster *et al*., 2002). In the present study, we used (for both pre-2007 and 2012 samples) the microplate assay for the total carboxylesteraces activity that was afterward developed as a rapid alternative, without the use of antiserum, for identifying the resistant *M. persicae* individuals (Devonshire, Devine & Moores, 1992). Twelve young (1–3 days old) adult females from each clone were tested with a total carboxylesterace activity test (Devonshire *et al*., 1992) and categorized as S, R1, R2, and R3 (susceptible, moderately, highly, and very highly resistant, respectively) according to the levels of enzymatic activity (Foster *et al*., 2002). Clones of known resistance profile were used as controls (US1L: S for pre-2007 samples; 4106A: R1, 5191A: R2 for 2012 samples). Data concerning the level of carboxylesterase activities and *kdr* resistance (see below) for most of the 2002–2005 clones were reported in a previous study (Margaritopoulos *et al*., 2007c), although they are included here for comparison purposes.

Detection of *E4*/*FE4* genes

Genomic DNA was extracted from individual aphids using the 'salting out' method (samples from 2002–2007) (Sunnucks & Hales, 1996) or the 'sodium hydroxide' method (2012 samples) (Malloch *et al*., 2006). For the samples collected in the years 2002–2007, a polymerase chain reaction (PCR)-based diagnostic (Field *et al*., 1999) was used to identify *E4* and *FE4* genes. To confirm the results, a number of clones was also examined using a restriction fragment length polymorphism-PCR method developed by Field, Crick & Devonshire (1996) and modified by Guillemaud *et al*. (2003). The Taqman assay devel-

oped by Anstead, Williamson & Denholm (2008) for *E4/FE4* allele discrimination was used in a MxPro Mx3005P machine (Stratagene) to test the clones collected in 2012. Clones of known resistance profile (4106A: *E4* and *FE4*, 5191A: *FE4*) were used as controls. The PCR was automatically analyzed in real time using the MXPRO QPCR, version 3.20 (Stratagene). A sub-sample of the 2012 clones was also examined with the methods of Field *et al*. (1996) or Field *et al*. (1999).

We also tested the clones for the presence of *kdr* mutation as this might affect the evolution of carboxylesterase based resistance in the field populations. The pre-2007 samples were examined with a PCR-based diagnostic (Guillemaud *et al*., 2003) and the 2012 samples were examined with a Taqman assay (Anstead *et al*., 2004).

Chromosome studies on 2012 samples

Cytogenetic data were collected for 29 different MLGs (as revealed by microsatellite genotyping) from the 2012 samplings. Chromosome spreads were obtained as described by Mandrioli & Manicardi (2013). Slides were then stained with a 150 ng mL^{-1} propidium iodide solution for 15 min at room temperature. Karyotype analysis was performed by measuring chromosome lengths on 75 metaphases using MicroMeasure (http://rydberg.biology.colostate.edu/MicroMeasure). The relative length of each chromosome was calculated to minimize problems as a result of different levels of chromosome condensation. Photographs of the metaphase were taken using a charge coupled device camera (Spot, Digital Instrument) and the SPOT software supplied with the camera and processed using PHOTOSHOP (Adobe Systems).

RESULTS

Microsatellite genotypes of 2012 samples

A total of 159 MLGs were identified among the 177 aphid clones that were analyzed with microsatellite DNA markers. The P_{SEX} values and the corresponding statistical *P* values suggested that all replicates of each multicopy MLG derived from asexual reproduction from a single zygote (data not shown). Statistics for the microsatellite data are provided in Table 2. Moderate to high allelic diversity (mean number of alleles per locus = 7.8–14.2) and high genotypic diversity (P_d = 0.625–0.960; total = 0.898) were observed. The genotypic diversity was comparable to that observed in the pre-2000 samples from peach or from herbaceous crops in northern Greece where the highest values were recorded (Blackman *et al*., 2007). The allelic diversity was similar or even higher (Margaritopoulos *et al*., 2007a). Although there is some variation among samples in the number of alleles, the allelic richness (number of alleles independent of sample size) was similar. A few cases of single locus HWE deviation were observed (nine out of the 60). Four cases were in NGPE (peach in North Greece; three associated with heterozygote deficiency and one with excess), three in NGTO (north Greece tobacco; one associated with heterozygote deficiency

Table 2. Number of aphids analyzed (*N*), number of multilocus genotypes (MLGs) (*G*), clonal diversity index ($P_d = G/N$), mean number of alleles (N_a), mean allelic richness (R_S, number of alleles independent of sample size), mean proportion of null alleles (A_N), and over all loci heterozygosity expected (H_{EXP}), heterozygosity observed (H_O), and inbreeding coefficient (F_{IS})

Sample*	*N*	*G*	P_d	N_a	R_s	A_N	H_O	H_E	F_{IS}†
CGPE	16	10	0.625	7.8	7.8	0.014	0.817	0.782	–0.044 (NS)
NGPE	50	48	0.960	13.8	7.7	0.031	0.750	0.804	0.067 (0.001)
NGPP	21	20	0.952	9.8	7.5	0.012	0.800	0.790	–0.012 (NS)
NGTO	77	69	0.896	14.2	7.7	0.018	0.804	0.785	–0.025 (NS)
NGWE	13	12	0.923	8.2	7.5	0.010	0.764	0.773	0.011 (NS)

*CGPE, central Greece, peach; NGPE, north Greece, peach; NGPP, north Greece, pepper; NGTO, north Greece, tobacco, NGWE, north Greece, weeds. Note that in each sample only one copy per MLG was used for the calculation of the genetic parameters.

†Probability for Hardy–Weinberg equilibrium deviation in brackets.

NS = not significant.

and two with excess) and one each in NGPP and NGWE (north Greece pepper and north Greece weeds, respectively; both associated with heterozygote deficiency). The multi-sample test (*U*-test) revealed significant deviation from HWE (involving heterozygote deficiency) in NGPE sample (north Greece Peach). Significant linkage disequilibrium was observed in 21 out of the 75 locus pairs examined. This is a higher number than expected by chance ($0.05 \times 75 = 3.75$). Most pairs (13 out of 15) with linkage disequilibrium were observed in NGTO. The Brookfield method (Brookfield, 1996) revealed very low frequencies of null alleles.

Total carboxylestarases activity and *E4*/*FE4* genes

In tests of total carboxylesterase activity of the 2012 samples, 18.7% and 81.3% of the MLGs were of R1 and R2/R3 resistance categories respectively (0.9% were R3) (Table 3). A high percentage (93.7%) of the MLGs were found to possess amplified *FE4* genes, and only 6.3% of these had both *E4* and *FE4* genes.

Similar frequencies for carboxylesterase genes and resistant phenotypes were found in the samples collected during the years 2002–2007 for which no information about the number of distinct genotypes is available. The percentage of aphids with amplified *FE4* genes ranged among years from 78.3% to 100% and those with both *E4* and *FE4* genes from 0.0% to 21.7% (pooled data per year; total for years 2002–2007: 96.9% *FE4* and 3.1% *E4* and *FE4*). Almost all of the clones with both *E4* and *FE4* genes were from tobacco. We never found a clone/MLG carrying only *E4* amplified genes (Table 3). Most of the clones were of R2/R3 category, with their percentages ranging from 56.6% to 91.7% (pooled data per year; total for years 2002–2007: 69.0% R2, 2.4% R3). Insecticide-susceptible aphids (S) were found only in two years at very low percentages (0.4% and 4.4%; pooled data per year) (Table 3).

Of the 1007 aphid clones examined for the life-cycle category, 981 had the *FE4* gene and 26 had both *E4* and *FE4* genes. Of the clones with *FE4*, 66.0% were sexual (holocyclic) and 34.0% were asexual (obligate parthenogens and intermediates). The clones with both *E4* and *FE4* genes were 30.8% sexual and 69.2% asexual. In the total sample, the proportions of sexual, obligate parthenogenetic, and intermediate clones were 65.0%, 32.7%, and 2.3%, respectively.

The *kdr* resistance was present in the aphid samples examined, with the total frequency of the R allele being 41.5% (pooled data over all years, $N = 1257$). The frequency of the R allele was higher in aphids from peach than in those from tobacco (57.6% versus 32.3%, pooled data over years).

Chromosome studies on 2012 samples

Twenty-four out of 29 different MLGs that were analyzed possessed twelve chromosomes ($2n = 12$). Of these, 18 had normal karyotype, although six had unusual karyotypes as a result of long autosomes in homozygous condition (MLGs 17 and 72), homozygous deletions (MLGs 24 and 41) and to heterozygous nonreciprocal partial translocation (MLGs 232 and 236). Four MLGs had 11 chromosomes as a result of a complete A1,3 translocation (MLGs 152 and 175) or loss of an A5 autosome (MLGs 1 and 10) and both these chromosomal mutations were heterozygous (Fig. 1, Table 4). We also found one MLG with an intra- and inter-individual chromosome mosaicism as a result of the presence of mitotic plates with 12 (64.0% of the observed plates), 13 (20.0%), and 15 (16.0%) chromosomes as a consequence of fissions involving autosomes A1 and A3 and the X chromosomes, all in heterozygous condition (Fig. 2, Table 4). MLGs with both *E4* and *FE4* genes were all of normal karyotype, whereas the *FE4* gene was present in MLGs of normal karyotype or in those with various chromosomal rearrangements. The life-cycle category was tested in 12 of the MLGs, four with unusual and eight with normal karyotypes. Of the MLGs with unusual karyotypes, three were anholocyclic (obligate parthenogens) (MLGs 1, 152, and 232) and one was holocyclic (sexual) (MlG 72). MLGs of normal karyotype were sexual or obligate parthenogens (Table 4).

DISCUSSION

The present study reports data on the carboxylesterase gene frequencies mostly for the tobacco-aphid populations from various regions in Greece collected during the years 2002–2007 and 2012, and provides a marked contrast to data obtained in the 1990s. The *FE4* gene was found to predominate in all years, whereas *E4* only occurred together with *FE4*.

The tobacco aphid has lost the *E4* amplified gene–A1,3 translocation association

Blackman *et al*. (1999) examined the frequencies of the *E4*/*FE4* carboxylesterase genes, the karyotype, and the number and distribution of loci with the amplified genes in more than 200 *M. persicae s.l.* clones that were collected from peach, tobacco, and other herbaceous crops in central and northern Greece in 1995–1996. In the total sample, 79.0% had normal karyotype and *FE4* amplified genes, and 19.6% had *E4* amplified genes associated with the A1,3 translocation (1.4% of the clones had both genes). When *nicotianae* is considered, 32.1% of the clones from tobacco had *E4* amplified genes and the

Table 3. Percentage (%) of *Myzus persicae* clones (before 2012) and multilocus genotypes (MLGs) (2012) with *E4* and/or *FE4* carboxylesterase genes and different levels of total carboxyesterase activity (S, R1, R2/R3)

Year	Region	Host	*N*†	*FE4*	*FE4+E4*	*N*	S	R1	R2/R3‡
2002	NG	Tobacco	12	100	0.00	12	0.00	16.67	83.33
2003	NG	Peach	11	100	0.00	11	0.00	45.45	54.55
	NG	Tobacco	6	66.67	33.33	6	0.00	33.33	66.67
	CG	Tobacco	15	66.67	33.33	14	0.00	0.00	100
	ECG*	Peach	9	100	0.00	8	0.00	50.00	50.00
		Total	41	82.93	17.07	39	0.00	28.21	71.79
2004	NG	Peach	28	96.43	3.57	17	0.00	0.00	100
	NG	Tobacco	29	68.97	31.03	23	0.00	17.39	82.61
	CG	Tobacco	20	60.00	40.00	17	0.00	0.00	100
	ECG*	Peach	27	100	0.00	25	0.00	16.00	84.00
	SG	Tobacco	16	50.00	50.00	15	0.00	0.00	100
		Total	120	78.33	21.67	97	0.00	8.25	91.75
2005	NG	Peach	20	100	0.00	19	5.26	36.84	57.89
	NG	Tobacco	57	100	0.00	55	5.45	34.55	60.00
	CG	Tobacco	30	96.67	3.33	28	0.00	21.43	78.57
	ECG*	Peach	28	100	0.00	24	4.17	58.33	37.50
	SG	Tobacco	34	100	0.00	33	6.06	48.48	45.45
		Total	169	99.41	0.59	159	4.40	38.99	56.60
2006	NG	Peach	128	100	0.00	125	0.00	24.00	76.00
	NG	Tobacco	201	99.50	0.50	195	0.00	26.67	73.33
	NG	Weeds	29	100	0.00	28	7.14	85.71	7.14
	CG	Weeds	1	100	0.00	1	0.00	0.00	100
	ECG*	Peach	79	100	0.00	77	0.00	36.36	63.64
	ECG*	Weeds	8	100	0.00	8	0.00	100	0.00
	SG*	Peach	19	100	0.00	18	0.00	27.78	72.22
	SG	Weeds	2	100	0.00	1	0.00	0.00	100
		Total	467	99.79	0.21	453	0.44	32.45	67.11
2007	NG	Peach	66	100	0.00	66	0.00	4.55	95.45
	NG	Tobacco	141	100	0.00	141	0.00	31.21	68.79
	NG	Weeds	1	100	0.00	1	0.00	0.00	100
	ECG*	Peach	56	100	0.00	56	0.00	23.21	76.79
	SG*	Peach	39	100	0.00	39	0.00	12.82	87.18
		Total	303	100	0.00	303	0.00	21.45	78.55
2012	NG	Peach	48 (50)	100	0.00	36	0.00	11.11	88.89
	NG	Pepper	20 (21)	100	0.00	13	0.00	7.69	92.31
	NG	Tobacco	69 (77)	88.41	11.59	42	0.00	33.33	66.67
	NG	Weeds	12 (13)	91.67	8.33	9	0.00	11.11	88.89
	CG*	Peach	10 (16)	90.00	10.00	7	0.00	0.00	100
		Total	159 (177)	93.71	6.29	107	0.00	18.69	81.31
	Grant Total		1271	96.46	3.54	1170	0.77	26.92	72.31

*Samples were from non tobacco-growing localities, all other samples were from tobacco-growing localities. For regional abbreviations, see Table 1.

†*N*, number of clones examined. For the 2012 samples, *N* denotes the number of different MLGs per group. The number in brackets denotes the number of clones genotyped using microsatellite DNA markers.

‡S, sensitive; R1, R2 and R3, moderately, highly, and very highly resistant, respectively. Of the clones before 2012 and the MLGs in 2012, 2.4% and 0.9%, respectively, were of the R3 resistant category.

A1,3 translocation. Data on Greek populations from peach, tobacco, and other crops (most of them were from tobacco-growing regions) collected in the late 1990s showed that all of the clones examined carried *FE4* genes (Denholm & Cox, 2002). This was a first indication of a genetic change in the Greek populations of *M. persicae s.l.* Our data suggest that this change has been fixed, at least in *nicotianae*, which constitutes the majority of the samples because, during the 10-year survey, the *E4* gene was found in

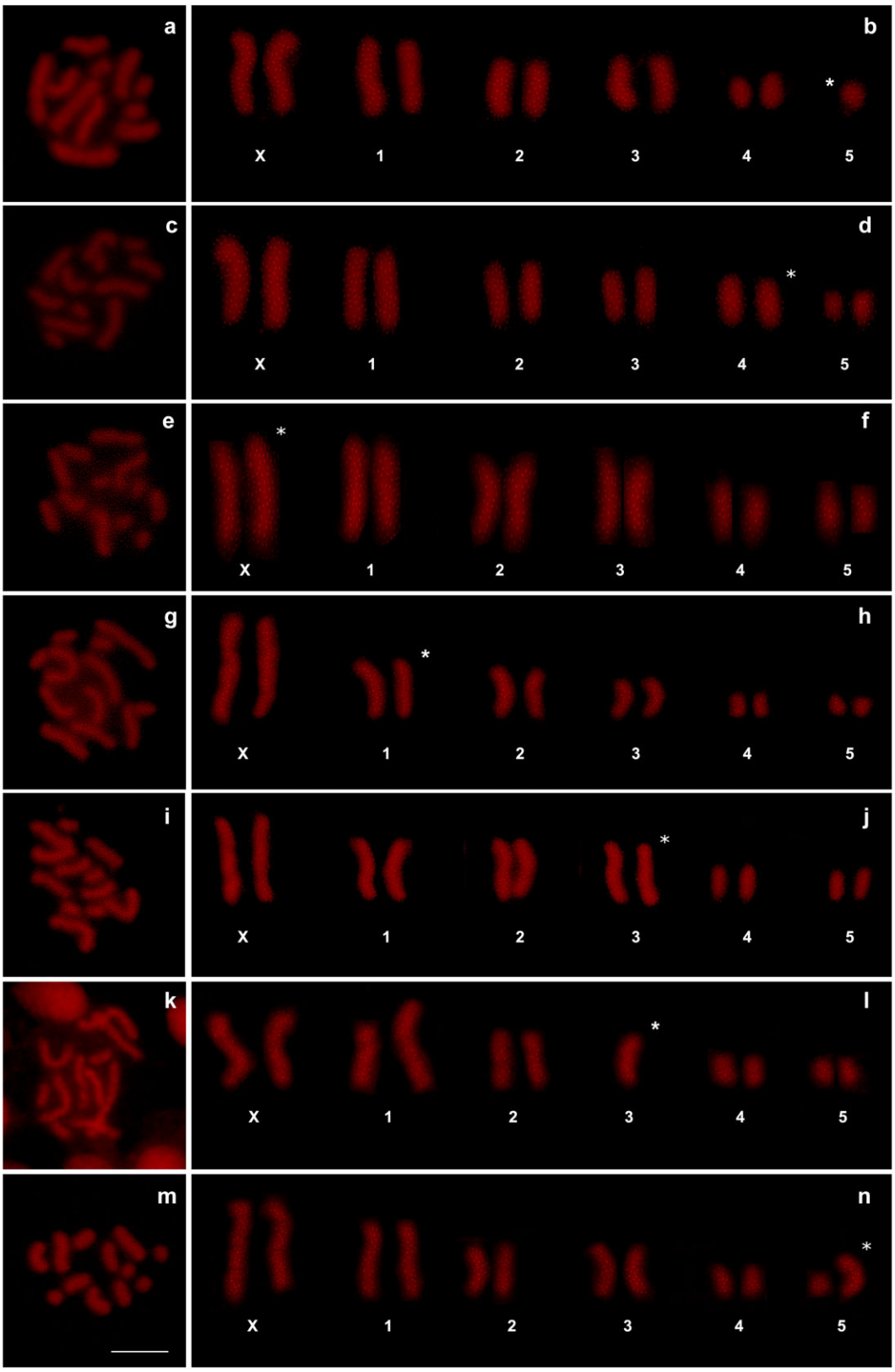

Figure 1. Propidium-stained metaphase chromosomes (a, c, e, g, i, k, m) and karyotypes (b, d, f, h, j, l, n) of multilocus genotypes (MLGs) 10 (a, b), 17 (c, d), 24 (e, f), 41 (g, h), 72 (i, j), 152 (k, l), and 236 (m, n) showing the different chromosomal rearrangements observed in the analyzed *Myzus persicae* populations. Scale bar = 10 μm.

Table 4. Karyotype variation, carboxylesterase genes, and carboxylesterase level in 29 *Myzus persicae* multilocus genotypes (MLGs) collected on crops and weeds (1, 3, 10, 90) from northern Greece (except MLG 72 from central Greece) in 2012

MLGs	Host	Chromosome number ($2n$)		Carboxylesterase genes	Carboxylesterase level	Life cycle
1	*Capsella bursa-pastoris*	11	Loss of A5	*FE4*	R2	Anholocyclic
3	*Lepidium draba*	12		*E4+FE4*	R2	Holocyclic
5	Peach	12		*FE4*	R2	NA
6	Peach	12		*FE4*	R2	Holocyclic
7	Peach	12, 13, 15		*FE4*	R2	NA
10	*Lepidium draba*	11	Loss of A5	*FE4*	R2	NA
17	Peach	12	Long A4	*FE4*	R2	NA
24	Peach	12	Short X	*FE4*	R2	NA
29	Peach	12		*FE4*	R2	NA
33	Peach	12		*FE4*	R2	NA
34	Peach	12		*FE4*	R2	NA
37	Peach	12		*FE4*	R2	NA
41	Peach	12	Short A1	*FE4*	R2	NA
61	Peach	12		*FE4*	R2	NA
63	Peach	12		*FE4*	R2	NA
72	Peach	12	Long A3	*FE4*	R2	Holocyclic
90	*Brassica oleracea*	12		*E4+FE4*	R2	Anholocyclic
107	Tobacco	12		*FE4*	R1	NA
109	Tobacco	12		*FE4*	R1	Anholocyclic
152	Tobacco	11	tA1,3	*FE4*	R2	Anholocyclic
175	Tobacco	11	tA1,3	*FE4*	R1	NA
183	Tobacco	12		*FE4*	R2	NA
184	Tobacco	12		*FE4*	R1	Anholocyclic
211	Pepper	12		*FE4*	R2	Anholocyclic
224	Tobacco	12		*E4+FE4*	R2	NA
232	Tobacco	12	tA5,X	*FE4*	R1	Anholocyclic
236	Tobacco	12	tA5,X	*FE4*	R1	NA
271	Pepper	12		*FE4*	R2	Holocyclic
279	Pepper	12		*FE4*	R2	Holocyclic

MLG 7 is a case of chromosomal mosaicism (both inter- and intra-individual variation).
loss of A5, loss of one A5 autosome; long A4, unusually long A4 autosomes; short X, unusually short X chromosomes; short A1, unusually short A1 chromosomes; long A3, unusually long A3 chromosomes; tA1,3, complete translocation of one A3 on one A1; tA5,X, partial translocation of a portion of one X chromosome on one A5 autosome.
Holocyclic, cyclical parthenogens (sexuals); anholocyclic, obligate parthenogens (asexuals); NA, data not available.

low frequencies and only in combination with the *FE4*. For *M. persicae s.s.*, we have much fewer samples, mainly from peach. Thus, it is difficult to infer about changes in this taxon, taking into account that *FE4* has always been associated with cyclical parthenogenetic populations. One problem in aphid population studies is 'clonal amplification' (i.e. the same genotype proliferates parthenogenetically). Inadequate sampling design or no information about the distinct genotypes in the material studied could bias the results. We tried to address this problem by collecting aphids from the primary host, where each aphid clone from different trees presumably originates from different sexually produced egg, and from secondary crops in northern Greece where the sexual populations predominate (Margaritopoulos *et al.*, 2002; Blackman *et al.*, 2007). In addition, in the year 2012, we genotyped the aphid material using microsatellite DNA markers. Thus, we consider that, during the present study, a high number of different aphid genotypes has been examined. The *E4*/*FE4* gene frequencies were examined with different methods in the 2002–2007 (PCR-based diagnostics) and 2012 samples (Taqman assay). Given that a subsample of the 2012 clones was also tested with the PCR-based diagnostics that provided the same results with the Taqman assay, the data generated with the two different methods are comparable.

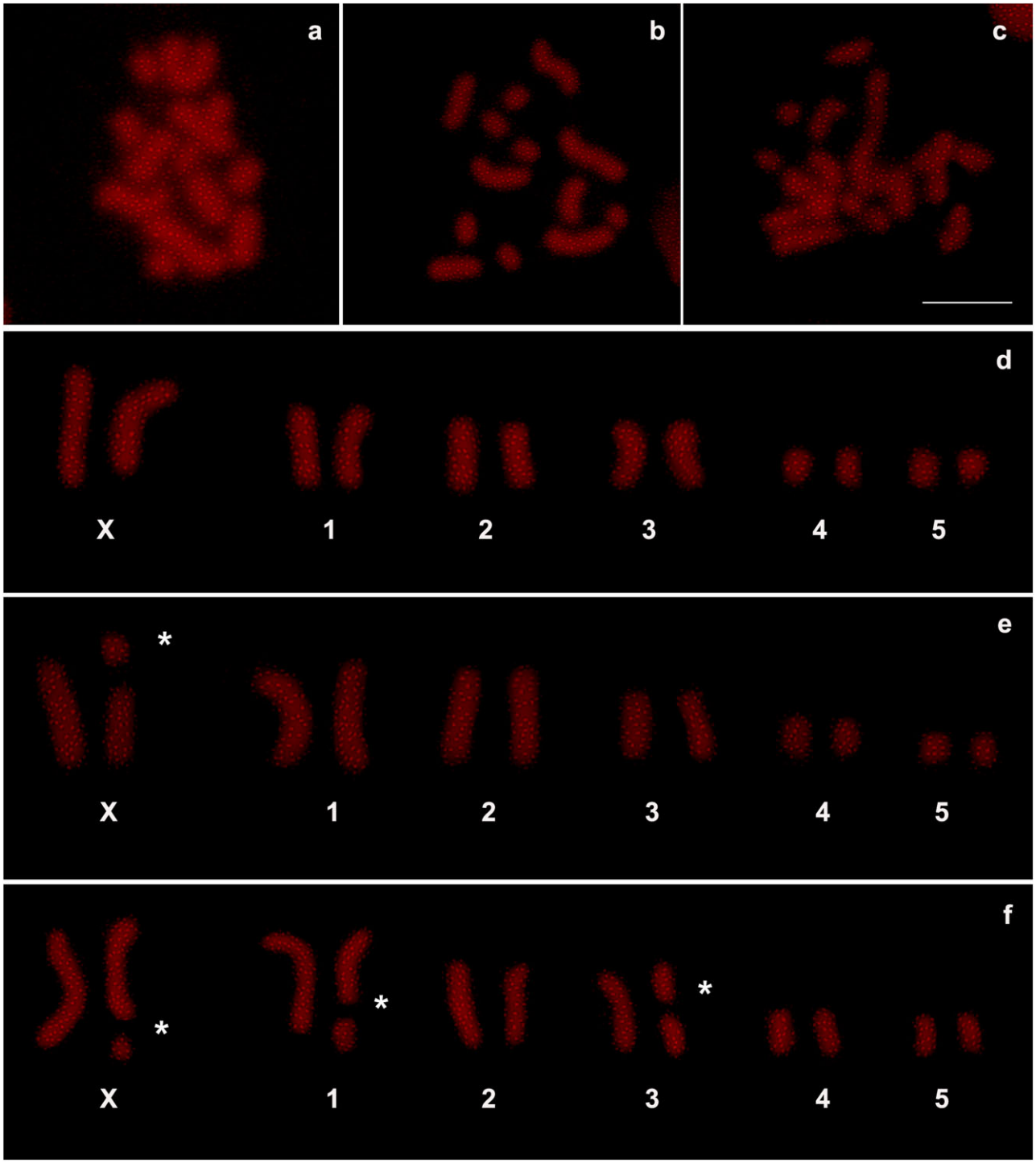

Figure 2. Propidium-stained metaphase chromosomes (a, b, c) and karyotypes (d, e, f) of multilocus genotype (MLG) 7 showing the occurrence of an intra- and inter-individual mosaicism as a result of the presence of chromosomal plates with 12 (a, d), 13 (b, e), and 15 (c, f) chromosomes. Scale bar = 10 μm.

The question that arises, is which mechanisms (related to aphid bio-ecology) are involved in the observed change and what are the responsible factors? This change in the genotype frequencies could be attributed to genetic drift or selection (or both). Selection in aphids is well documented and there are many examples demonstrating the effect of this phenomenon on the genotype frequencies either in short (one

growing season) or long (through years) time scales. For example, host-plant is an important selective factor, as has been highlighted in various studies on host-adapted aphid genotypes, races or subspecies (e.g. *Acyrthosiphon pisum* (Harris): Peccoud *et al*., 2009; *M. persicae*: Vorburger, 2006; Margaritopoulos *et al*., 2007a). The adaptation to a particular host and the emergence of host-adapted taxa might be a rather slow evolutionary process. On the other hand, intense insecticide application is a strong evolutionary force that provide the opportunity to examine evolution in perceptible timescales. Indeed, both short- and long-term studies have shown that insecticides dramatically change the frequency of the aphid genotypes and the genetic structure of the populations (Fenton *et al*., 2005, 2010; Zamoum *et al*., 2005; Kasprowicz *et al*., 2008). In a study similar to ours conducted in UK populations, Field & Foster (2002) reported a shift from clones with high *E4* gene copy numbers (mostly R2/R3 resistant category in 1997) to clones with lower copy numbers of *FE4* (mostly R1 resistant category in 1998–2000). The results were explained based on the possible effect of changes in insecticide use and therefore a different selection pressure was applied to the aphid populations. It was also concluded that there may be a fitness cost associated with having amplified carboxylesterase genes. This may also be the case for the change observed in the aphid populations in Greece. After the mid-1990s, the farmers reduced the use of organophosphates, carbamates, and pyrethroids against *M. persicae* especially on tobacco crops, and, to a great extent, neonicotinoids replaced the former aphicides. However, pyrethroids are used in peach orchards against other pests. This might be the reason for the presence of *kdr* resistance through the years and the higher frequency of *kdr* allele in samples from peach compared to those from tobacco. The extensive use of neonicotinoids may select genotypes with different resistance and biological traits, although pyrethroid resistance might have also played a role as association with *E4* or *EF4* and *kdr* has been observed in other countries (e.g. UK; Field & Foster, 2002). The change from high (*E4*, R2/R3) to low resistance categories (*FE4*, R1) found in UK populations (Field & Foster, 2002) does not hold in our samples because most of the clones and MLGs were highly resistant (R2/R3; although only a few were R3), and this is consistent with a previous survey (Margaritopoulos *et al*., 2007c). The high frequency of carboxylesterase-based resistance for more than a decade, despite any possible fitness-cost, could be attributed to milder winters compared to western Europe and to the sexual reproducing populations. Mild winters lead to weaker selection against asexual resistant genotypes that suffer from fitness costs (e.g. winter survival, movement from deteriorating leaves; Foster *et al*., 2002; Fenton *et al*., 2010) and sexual genotypes overwinter at the cold-tolerant diapause egg stage on peach that act as a 'refuge' (for a discussion, see Margaritopoulos *et al*., 2007c). It appears, therefore, that any fitness-cost related to esterase-based resistance is not the major reason for the change from *E4* to *FE4* genes observed in the tobacco-aphid. Whatever the selection agents related to changes in the crop-protection practices and the aphicides used, the possible comparative advantages of the *FE4* genotypes need to be researched further. Clonal competition as a result of factors other than insecticide resistance mechanisms and their pleiotropic effects is also well documented in aphids (e.g. differences in the susceptibility to parasitoids and predators; Henter & Via, 1995; Braendle & Weisser, 2001). It is worth noting that stochastic events (including genetic drift, bottlenecks) might contribute to the change observed in addition to selection. In the large populations, typical of aphid pests, drift is expected to play a negligible role (Vorburger, 2006); however, for a turnover in gene and genotype frequencies in the tansy aphid *Macrosiphoniella tanacetaria* (Kaltenbach), see also Loxdale *et al*. (2011a). The current data, as well as those reported in previous studies on Greek populations, argue for clonal selection (Margaritopoulos *et al*., 2007a, 2007c, 2009) and also provide good indications about the effect of insecticides on population structuring. HWE deviations associated with heterozygote deficiency in sexual populations of *M. persicae s.l*. from peach, an intense chemically protected cultivation, have been attributed mostly to aphid inbreeding as a result of the selection of resistant genotypes (Fenton *et al*., 2003). However, various reasons have been discussed in other studies (e.g. *R. padi*; Delmotte *et al*., 2002). Such deviations have been recorded in the present study on peach samples (northern Greece) and in some populations from the same or neighbouring regions in previous studies (sexual populations on herbaceous crops in northern Greece: Margaritopoulos *et al*., 2007a; populations on peach in northern and central-eastern Greece: Margaritopoulos *et al*., 2009), which might denote selection events.

Studies on *M. persicae s.l.* populations from other Mediterranean countries have also shown that *FE4* genes predominate. In Italy, all of the clones examined [Bizzaro *et al*. (2005): 22 clones from peach; Rivi *et al*. (2013): 38 clones from peach, tobacco and other crops], all (> 200) but one aphid individuals from peach trees in southern France (Guillemaud *et al*., 2003) and 95% of the aphids (> 600) from oilseed rape from France (Zamoum *et al*., 2005) possessed *FE4* genes. By contrast, amplified *E4* genes predominated in samples of the tobacco aphid from the USA (Srigiriraju *et al*., 2009) where *FE4* genes were also found in a number of samples alone or together with *E4*.

A change, parallel to that occurring in carboxylesterase gene frequencies, was also observed in the frequency of certain karyotypic variants. Blackman *et al*. (1999) reported that 21.0% of the Greek clones (32.1% of the clones from tobacco), which were collected in 1995–1996, had the A1-3 translocation that was at that time invariably associated with amplified *E4*. Another study focusing on the distribution of common MLGs in various hosts and regions in Greece in 1998–1999 reported that 30.0% of the clones (36.2% of those from tobacco) had the A1-3 translocation. This translocation was found in three clones from peach and in three out of the six commonest and most widespread MLGs (Blackman *et al*., 2007). The A1,3 translocation was also reported by Denholm & Cox (2002) in Greek samples collected 1998–1999, although it was associated only with *FE4*. In the course of the present screening for karyotypic variants, we found for the first time in *M. persicae*, clones from tobacco with a translocation of a complete autosome A3 onto A1, so that the chromosome number was 11 instead of the normal $2n = 12$. The A1-3 translocation that was common in past surveys, was totally absent in the present screening.

Clones with the partial A1,3 translocation but with *FE4* rather than *E4* genes have been found recently for the first time in southern Italy, where it was the commonest chromosomal rearrangement (Rivi *et al*., 2012). Given that dispersal of aphid genotypes between the two countries has been documented (Blackman *et al*., 2007), it is of particular interest to determine further the current situation in Greece.

New chromosomal rearrangements

In addition to the novel A1-3 translocation, we found for the first time a translocation involving a portion of one X chromosome translocated on an autosome A5. All of the chromosomal translocations, losses or fissions of autosomes observed in the present study were found in samples from tobacco or other host-plants in tobacco-growing regions. They were all heterozygous and therefore possibly lethal in homozygous condition, in accordance with previous data (Blackman *et al*., 1999; 2007). By contrast to the previous survey in which all clones with *FE4* were karyotypically normal (Blackman *et al*., 1999), several *FE4* clones possessed rearranged karyotypes (including a clone with intra- and inter-individual mosaicism).

Most of the karyotype changes observed in recent studies in Italy have also occurred in populations on tobacco plants or on peach in tobacco-growing regions (Rivi *et al*., 2012; 2013). This suggests that the observed chromosomal rearrangements in the present study could possibly be influenced by the clastogenic effects of nicotine (Trivedi, Dave & Adhvaryu, 1990; Trivedi, Dave & Adhvaryu, 1993; for further discussion and references on clastogenic effects of nicotine, see Rivi *et al*., 2012). We have found clones with rearranged chromosomes on host plants other than tobacco, although these were also from tobacco-growing localities in northern Greece where *nicotianae* predominates (Margaritopoulos *et al*., 2000; 2007a). The fission of chromosomes (e.g. by tobacco mutagens) may be lethal in organisms with monocentric chromosomes (localized centromeres) because chromosomal fragments tend to be eliminated during mitosis and meiosis. By contrast, aphids can cope with this as a result of the holocentric nature of their chromosomes where centromeric activity is dispersed along the length of each chromosome. As a consequence, chromosome fragments can move to the daughter cells at successive cell divisions. Chromosomal rearrangements that survive mitosis might increase in frequency during the parthenogenetic phase of the aphid life cycle, although they may be selected against or eliminated in the meiotic process during the sexual reproduction events. Populations or species that have partially or completely lost the sexual reproduction show exceptional karyotype variations (Blackman, 1980; Blackman, Spence & Normark, 2000). In the present study, three of the four MLGs with known life cycle and unusual karyotype were asexual, suggesting a long-term persistence of these karyotype variants. However, some chromosomal rearrangements can pass through meiosis (Blackman & Takada, 1977; Spence & Blackman, 2000) so that karyotype variations can also be observed in sexual aphid clones such as those found on peach in the present and in previous studies (Rivi *et al*., 2012; 2013).

A case of gene flow among cyclical and functional parthenogens?

Another interesting point is how the *FE4* genes passed from the sexual reproducing populations (from which it is believed to originate) to asexual genotypes (functional parthenogens) or how both *E4* and *FE4* genes are present in sexual and asexual genotypes. *FE4* genes have also passed to aphids with the A1,3 translocation (or other chromosomal rearrangements). Blackman *et al*. (1999) found that three clones from pepper from central eastern Greece with the A1,3 translocation had both amplified genes. Such clones have been obtained previously in laboratory crosses (Blackman *et al*., 1996) but this was the first report in field-collected aphids. Srigiriraju *et al*. (2009) found five colonies of the tobacco aphid in the USA with both *E4* and *FE4* amplified genes and Field & Foster (2002) two clones of *M. persicae s.l*. in England. In addition, *FE4* genes associated with the A1,3 translocation has been reported in Greek populations of *M. persicae s.l*.

by Denholm & Cox (2002) in pre-2000 samples and recently in central-southern Italy by Rivi *et al*. (2013). These are indications of gene flow between sexually reproducing *FE4* genotypes and functional parthenogens carrying the translocation and/or the *E4* genes. Such cases of gene flow have been suggested or proved for field collected clones of various aphid species (e.g. *M. persicae s.l.*: Vorburger, Lancaster & Sunnucks, 2003; Margaritopoulos *et al*., 2007a; *R. padi*: Halkett *et al*., 2008; *Sitobion avenae* (Fabricius): Simon *et al*., 1996). Northern Greece, where the majority of the peach orchards are located, appears to be a suitable place for such evolutionary events because genotypes investing in different reproductive strategies coexist on tobacco and other crops, usually adjacent to the peach orchards where sexual reproduction takes place (Margaritopoulos *et al*., 2002; Blackman *et al*., 2007).

Conclusions

The present study provides evidence about rapid evolution in *nicotianae*, which can be considered as a species 'in the making'. Evolutionary changes over short periods require strong selection pressure and agricultural practices are good candidates (Fenton *et al*., 2010). As Loxdale, Lushai & Harvey (2011b) have also mentioned in their review about specialization in animals, 'agricultural and medical-veterinary systems are the best areas exemplifying rapid evolution'. Resistance to organophosphates in tobacco aphids was first reported in holocyclic populations in northern Greece in the mid-1980s (Blackman & Eastop, 2007). This may be where introgression of carboxylesterase genes into *nicotianae* occurred and then spread to other populations. The amplified *E4* genes were present in the tobacco aphid populations surveyed in 1995–1996 and, within approximately 5 years, these genes were absent or hardly found and only in combination with the amplified *FE4* genes. Whatever the exact mechanism for this change (although the most probable factor is the changes in the agrochemicals used), *nicotianae* appears to possess the genetic material to evolve further and to adapt in diverse conditions. This may be aided by the sexual reproduction and the high genetic diversity of the aphid in Greece and, as Loxdale (2010) pointed out in his review, rapid genetic changes are further enhanced in insects by their fast rates of reproduction. This is more pronounced in aphids that combine one sexual event and many parthenogenetic generations in one year. We provided also evidence about possible effects of nicotine, coupled with the holocentric nature of aphid chromosomes, in the creation of novel chromosomal rearrangements, although whether these are adaptive remains to be found.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Sampling sites in Greece. 1, Argos; 2, Prosymni; 3, Amfiklia; 4, Lehonia; 5, Velestino; 6, Tirnavos; 7, Karditsa; 8, Milia; 9, Katerini; 10, Velvendos; 11, Meliki; 12, Kria Vrisi.