

CHAPTER 2



1 INTRODUCTION

POPULATION STRUCTURE USING MICROSATELLITE MARKERS

1.1 GENERAL CONSIDERATIONS ABOUT GENETIC MARKERS

The main tools used to study the genetic variation within populations or between populations are represented by genetic markers. A genetic marker is any visible character or otherwise assayable phenotype, for which alleles at individual loci segregate in a Mendelian manner. Genetic markers can also be used to study the genetics of organisms at the level of single genes. To date, researchers applied different molecular approaches (e.g. microsatellites or minisatellites, RAPD, RFLP and AFLP) in order to deepen studies about genetic population structure. To analyze the genetic structure of a population means to compare the gene pool of a specific population with the population hence it derives and/or with other populations. The comparison will be made by analyzing the distribution of allele frequencies and studying the different haplotypes. Furthermore, the molecular markers have a wide variety of applications in both basic research and applied improvement programs for example in the study of ecology and evolution (breeding, population structure and gene flow, fitness, etc.) (Hartwell et.al., 2004).

The development of the discipline of genetics would not have been possible without genetic markers such as the visible characters in peas and *Drosophila*.

It was not until the early 1970s that biochemical genetic markers such as terpenes and allozymes were developed. These biochemical markers were applied to an array of research problems, most notably the study of amounts and patterns of genetic variation in natural populations and the characterization of mating systems (Hartwell et.al., 2004). However a major limitation of biochemical markers, is that there are only a few number of different marker loci; therefore, genetic information obtained from such markers may not be very representative of genes throughout the genome. The limitation in the number of markers was overcome beginning in the 1980s with the development of molecular or DNA-based genetic markers (Amos et.al., 1996). The molecular markers allow to identify rare alleles ensuring the protection of biodiversity, to carry on the identity of the species or identify potential errors of classification. For instance, in taxonomic studies, the markers are also important for specimens classifications and analysis of phylogenetic relationships (Page et.al., 1998).

In recent years many molecular biology techniques have been developed allowing to replace the phenotypic markers with those to be used in the molecular characterization of the species and variety. In this regard, one of the most interesting targets to be achieved in the agronomic field is the development of genetic linkage maps that allow to associate a certain characteristic to a particular polymorphic marker.

Therefore, a genetic marker is a characteristic of the organisms that varies within populations and which is determined by the genes and not from the environment, thus being inheritable (Johnson et.al., 2000).

The requirements of an ideal genetic marker, listed below, are basic to discriminate a marker on the other :

- 1 . dominance / co-dominance;
- 2 . criterion of inheritance;
- 3 . recombination.

The degree of variation will depend on the specific question to which you want to answer, and also from population and species that we intend to study (Johnson et. al., 2003).

1.1.1 A Direct Look At Microsatellites

The genomes of most of the organisms are loaded with loci defined by repeated sequences. The repeating unit can be as short as a single base pair or as long as tens of kilo-bases. The number of repeats can vary between two and thousands (Hartwell et.al., 2004). Among repeated sequences, the most useful for genetic population analyses are **microsatellites**, also known as simple sequence repeats (SSR) or short tandem repeats (STR) (Fig1.1) (Oliveira et.al., 2006).

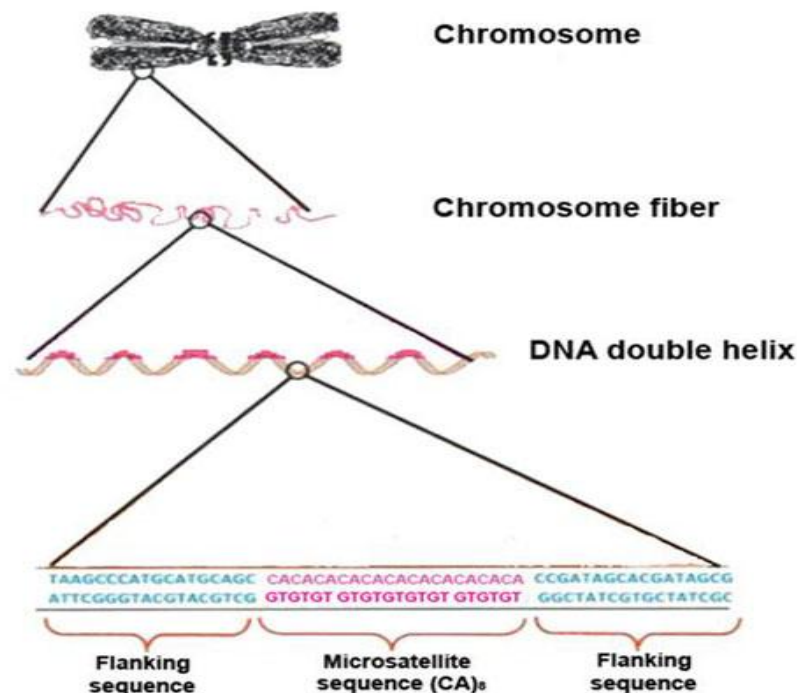


Fig 1.1. Schematic representation of a microsatellite locus from (Aquatic Stock Improvement Company (ASiCo)).

Especially, microsatellites, are not-coding repetitive DNA regions composed of small motifs of 1 to 6 nucleotides repeated in tandem, which are widespread in both eukaryotic and prokaryotic genomes (Field et.al., 1998; Tóth et. al., 2000). Microsatellites have a particular attribute in that they suffer higher rates of mutation than the rest of the genome, here's why they are widely used as a markers (Jarne et.al., 1996).

Moreover, they are classified according to the type of repeat sequence as perfect, imperfect, interrupted or composite. In a perfect microsatellite the repeat sequence is not interrupted by any base not belonging to the motif (e.g. TATATATATATATATA) while in an imperfect microsatellite there is a pair of bases between the repeated motifs that does not match the motif sequence (e.g. TATATATACTATATA). In the case of an interrupted microsatellite there is a small sequence within the repeated sequence that does not match the motif sequence (e.g. TATATACGTGTATATATATA) while in a composite microsatellite the sequence contains two adjacent distinctive sequence-repeats(e.g. TATATATATAGTGTGTGT GT) (Eder Jorge Oliveira et.al., 2006).

It is known that the mutation rate of microsatellites is much higher than that of other parts of the genome, ranging from 10^{-2} to 10^{-6} nucleotides per locus per generation. A small number of genes naturally contain microsatellite sequences with triplet repeat units within their coding regions.

The propensity of the microsatellite sequences to change in size from one generation to the next can produce mutant alleles with drastic effects on phenotype. Microsatellites can arise spontaneously from random events that initially produce a short repeated sequence with four to five repeat units.

Once a short microsatellite mutates into existence, however, it can expand into a longer sequence by the process shown in Fig 1.2 (Hartwell, et.al., 2004). Moreover, different studies have showed that faulty DNA replication is the main mutational mechanism (Fig. 1.2). Because the same short homologous unit (CA, see in example) is repeated over and over again, DNA polymerase may develop a stutter during replication, that is, it may slip and make a second copy of the same di-nucleotide, or skip over a di-nucleotide. Microsatellites are thus highly polymorphic in the number of repeats they carry, with many alleles distinguishable at each microsatellite locus.

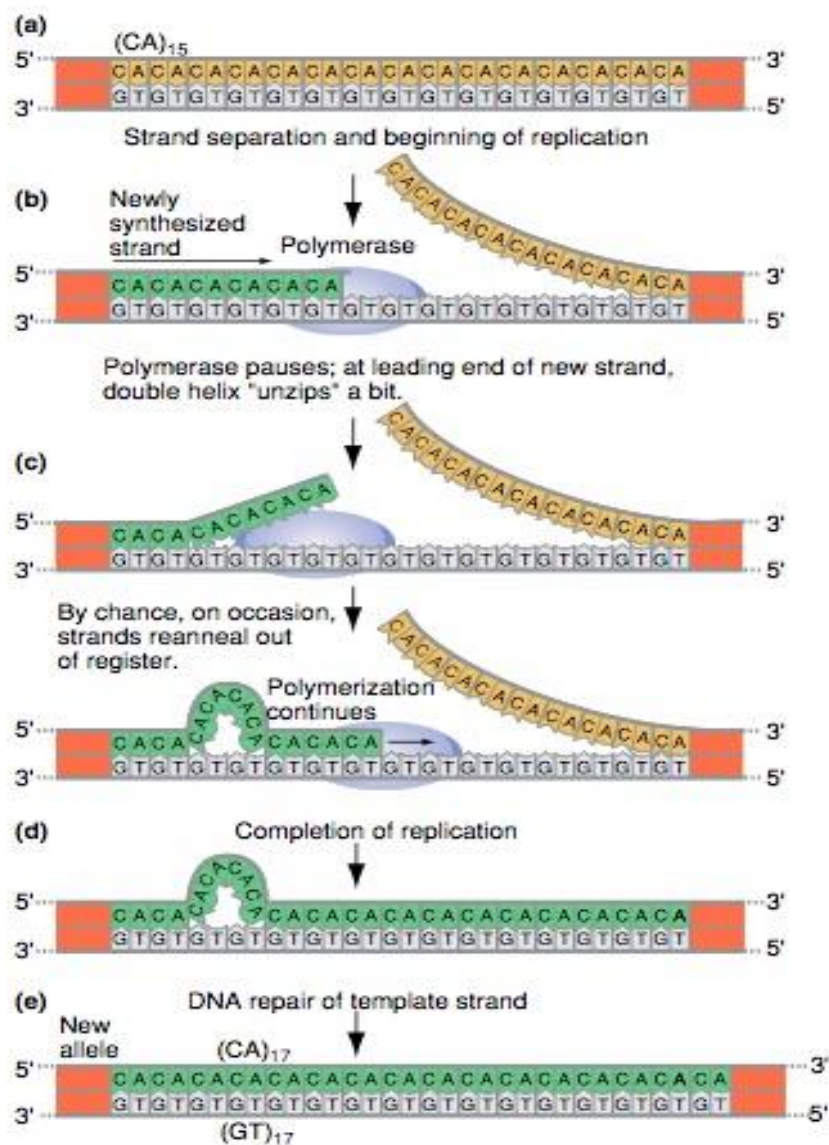


Fig 1.2. Faulty replication induces polymorphic microsatellites. Image from (Hartwell et al., 2004, Chapter 11). The microsatellite is consisting of 15 tandem repeats of the C-A dinucleotide sequence.

Another important feature of microsatellites is that an homozygous microsatellite locus has the same number of repeats on both homologous chromosomes, whereas a heterozygous microsatellite locus has a different number of repeats for each allele, e.g. one allele can contain 9 repeats and the other 10. However, at the same locus the population as a whole usually contains several alleles each with a different number of repeats, which means that microsatellite markers are very useful for discriminating different individuals. Hence, the high discriminating power of microsatellites is an

important characteristic which justifies their use in population genetic studies and in forensic science (Oliveira et.al., 2006).

Therefore, in the past few years, microsatellites have attracted the attention for a number of reasons, mainly for their practicability and ease of use in studies of population genetics, and for genotyping and paternity analysis (Wright et.al, 1994). According to Heywood and Iriondo (2003), microsatellite markers provide relevant information for identifying conservation units and for investigating the genetic processes that take place in populations such as patterns of gene flow, generation of genetic neighborhoods and the incidence of genetic drift. Currently, microsatellite markers are commonly employed for the analysis of population genetic structure of both wild (Zucchi et.al., 2003) and crop species (Pinto et al., 2003a, b) because of their co-dominant nature and high informativeness.

Wildlife populations in the 21st century faced with many anthropogenic threats to their persistence. Climate change, habitat fragmentation and loss, disturbance, pollution, competition from invasive species, over harvesting and novel pathogens all constitute extinction risks and major challenges to evolutionarily sustainable wildlife management (Ashley et.al., 2003). The potentially detrimental evolutionary effects of selective harvesting are more subtle and difficult to detect. They are also likely to be widespread and deleterious to the long-term viability of harvested populations. Therefore, molecular markers can be used as a tool to study how selective harvesting by humans can impact the evolutionary dynamics of wildlife populations but also, they can be used to assess the genetic structure of wildlife populations, and thereby assist in the prediction of genetic impacts by delineating evolutionarily meaningful management units. Finally, genetic markers can be used for monitoring genetic diversity and changes in effective population size and breeding systems (Coltman 2008).

1.1.2 *Genesis Of Microsatellites*

Microsatellites differ from most DNA sequences in that they have a distinct “birth.” These repeats likely originate from an initial expansion of an existing sequence motif, perhaps from a replication slippage or unequal-crossing-over event, although few cases of microsatellite births are documented (Schlötterer et.al., 2000).

The origin of microsatellites in genomes appears to be non random, with an imbalance between the mechanisms that promote and those that prevent the microsatellites initiation (Bhargava et.al., 2010). Currently, there are two non-mutually exclusive hypotheses to explain the origin of microsatellites:

- *de novo* microsatellites (Messier et.al., 1996) - suggests that the birth of microsatellites

was a consequence of the creation of a *proto-microsatellite*, a short region of as few as 3 or 4 repeated units within cryptically simple sequences, which are defined as a scramble of repetitive motifs lacking a clear tandem arrangement. Proto-microsatellites were originated from base substitutions (Buschiazzo et.al., 2006). Once a proto-microsatellite is initiated, maintenance and multiplication are favoured by its propensity to undergo strand slippage during replication and, depending primarily on the repeat motif, its capacity to form unusual DNA conformations and participate in recombination and transposition events. The number of repeat units correlates positively with the mutability of the microsatellite, but the minimum repeat number required for strand slippage or other mechanisms of microsatellite mutation is debatable (Jentzsch et.al., 2008). Adopted microsatellites (Wilder et.al., 2001) suggests that microsatellites arise from other genomic regions via transposable elements.

The transposable elements might contain one or more sites that are predisposed to microsatellite formation and hence favour the dispersal of microsatellites in genomes. Transposable elements can be divided into two main classes based on their mechanisms of movement: class I (retrovirus-like transposons) and class II (so called cut and paste transposons). Both of these elements can leave traces of their presence and movement during the transposition process across DNA sequences, which resemble microsatellites, especially poly A arrays. A poly A tail is added to the 3' end of class I retrotransposons after mRNA transcription, which then gets inserted together with the transposed sequence into the new position. Retrotransposons can also contain other microsatellite-like stretches, dinucleotide and tetranucleotide repeats, within their sequences (Akemi Hoshino et.al., 2012). Class II transposons preferentially insert into certain DNA sequences, which can be either inverted repeats or tandem repeat sequences. This suggests a reciprocal association in which microsatellites act as 'retroposition navigator sequences,' while retrotransposons generate more microsatellites during their dispersion throughout the genome. An example of a retrotransposon - mediated microsatellite genesis in humans is the origin of A/T rich microsatellites with motifs ranging from one to six nucleotides in length from *Alu* elements (Jentzsch et.al., 2008). For instance, Ramsay et.al., (1999) analyzed microsatellite flanking sequences in hops and showed that a high proportion of clones were homologous to known transposons. An association was found between the repetitive dispersed element R173 and the transposons BARE-1, WIS2-1A and PREM1. The microsatellites found in Ramsey's study were of two types, those with single sequences in the flanking region and those associated with retrotransposons and other repetitive dispersed elements. Three subtypes compose the second type: a) those positioned at terminal 3' of the transposon with a single sequence at the other terminal; b) those positioned at terminal 5'; and c) those in which the internal sequence of the transposon is homologous in both flanking regions.

Finally, different repeated analyses of sequencing and hybridization on mammalian genomes, have showed tandem repeats of DNA sequences with no apparent function scattered throughout the genome. The size of the repeating units ranges from two nucleotides (CACACACA . . .) to 20 kb or more, and the number of tandem repeats varies from two to several hundred. Hence, the mechanism by which these tandem repeats originate may be different for loci having very short repeat units as compared with loci having longer repeat units. Tandem repeats of short di- or trinucleotides can originate through random changes in nonfunctional sequences. By contrast, the initial duplication of larger repeat units is likely to be a consequence of unequal crossing-over. This event could create drastic changes such as the loss or gain of a large number of repeats (Hartwell et al., 2004) Fig 1.3.

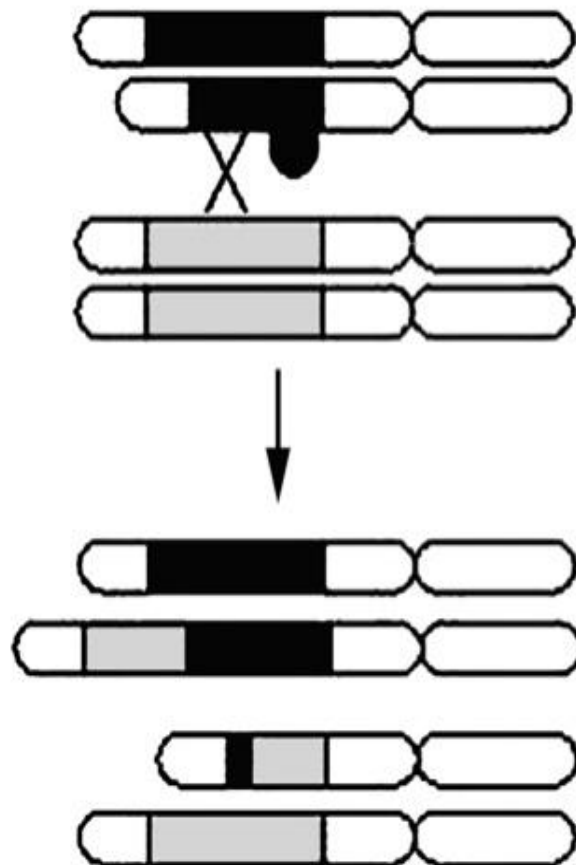


Fig 1.3. Unequal crossing-over between homologous chromosomes. Black and gray regions are microsatellite repeat sequences (Modified from Eder Jorge Oliveira et.al., 2006).

1.1.3 Function And Genetic Power Of Microsatellites

Microsatellites can have either a neutral effect on the genome or perform important functions in particular species. In medical research, microsatellites are often used to follow the variability of a specific chromosomal region in patients which suffer of heredity diseases. Therefore, the markers with a specific length that are never present in healthy patients but only in patients with diseases, could suggest a high probability that these specific microsatellites are in linkage with mutant genes of diseases, as described in *diabetes mellitus* (Chioramonte et.al., 2002). Not only, these molecular markers are also known to be related to pathogenicity and genomic variability in microorganisms and many examples of microsatellites associated with the modulation of microbial gene expression have been identified (Jackson et.al., 1997; Field et.al., 1998; Saunders et.al., 1998). The Adaptive Peaks Theory (Wright 1931; 1932) and the fact that the frequency of a microsatellite allele represents a maximum local adaptive value for the population suggests that the majority of mutations generating new alleles result in gene variants of lower local adaptive value.

A number of authors have suggested another function for microsatellites and showed that di-nucleotide repeats could act as recombination hot spots (Trecó et.al., 1986; Bailey et.al., 1998). This microsatellite function allows populations to recover genetic variation lost through genetic drift and to rapidly adjust to evolutionary demands (Rosenberg et.al., 1994).

One of the most interesting fields in which microsatellites are used is the research of polymorphisms. Polymorphism is a genetic variation within a single species and, in the case of microsatellites, it consists in the variation of the number of repeated monomers. The high polymorphism of these nuclear markers is a powerful means of investigation in the analysis of intraspecific variability on geographic scale. Moreover, the flanking regions of the sequences repeats - on the contrary- tend to be much more conserved; for this reason the microsatellites can be employed in order to study the genetic differentiation within species, and also between species phylogenetically close (Goldstein et.al., 1999). Over the last decade microsatellites have played a role more and more important in studies concerning the analysis of genetic variability among plants. The first analyses date back to the late 1980s when polymorphic microsatellites were found in different species of plants such as barley (Weising et.al., 2010), soybean (Akkaya et.al., 1992) and rice (Panaud et.al., 1995). Using microsatellites may allow to save time, resources and energy and also provides an objective method of analysis and evaluation, based on the study of features not detectable at the phenotypic level but only at the genotypic one (Leigh et al., 2003). Microsatellites were also used to distinguish varieties of potato (*Solanum tuberosum*) (McGregor et al., 2000), rice (Garland et al., 1999), the screw (Ortiz et al., 2004) and olive (Bandelj et al., 2004) but also in

animals, for instance in tsetse fly *Glossina pallidipes* (Ouma et.al.,2003) or in the silkworm *Bombix mori* (Damodar et.al.,1999) and finally in marine fishes (DeWoody et.al.,2005). After all, in animals microsatellites are often used to screening the different populations and to examine the evolutionary relationship between related species.

One of the most interesting targets to be achieved in the field of plant - genetics is the development of genetic linkage maps. Indeed, the ability to identify closely linked markers at loci with favourable phenotypic effects can simplify the genetic improvement programs for quantitative traits, allowing selection based on genotypic rather than phenotypic.

In this case the microsatellites have identified in tomato and also in *Arabidopsis* a specific repeat leucine-rich, typical of the resistance genes (Pan et al., 2000). In soybean, a gene Rhg1 for resistance to a nematode was identified (Cervigni et al., 2004). Therefore, microsatellites are extremely useful for estimating genetic population parameters as (i) population structure, (ii) parentage and paternity analysis and (iii) gene flow.

1.1.4 Genetic Structure Of Populations by Microsatellites

As discussed before, microsatellites are the most suitable markers for study the population structure, due in part to the abundance of alleles per locus and to their ability to distinguish heterozygotes from homozygotes. The populations of most, if not all, species show some levels of genetic structuring, which may be due to a variety of nonmutually exclusive agents (Balloux et.al., 2002). Genetic structuring reflects the number of alleles exchanged between populations, it has major consequences on the genetic composition of individuals themselves. Hence, the structure is assessed by studying the frequency and interaction of alleles and genes in populations. It has revolutionized many fields of evolutionary biology over the last 30 years and represents the essence of the modern evolutionary synthesis. The allelic frequency in populations can change spatially and temporally under the influence of various evolutionary process such as natural selection, genetic drift, mutation, gene flow, and mating system. These parameters are included in the theory of Hardy-Weinberg equilibrium, enunciated in 1908, and are able to influence the composition of the population gene pool. Comparative analyses of spatial and temporal pattern in allelic frequency provide an important entry point to identify the evolutionary forces that gave rise to them. However, the fundamental power and premise behind population genetics, is that one can compare allele frequencies at selectively neutral marker loci to estimate gene flow, under responsible assumption about the rate of drift, mutation, and mating system. Gene flow is a parameter of critical importance in studies related to wildlife conservation,

insect resistance management, pest management, pest eradication programs. Understanding gene flow and its effects, is central to many fields of research including populations genetics, populations ecology, conservation biology and epidemiology. Gene flow is a collective term that include all mechanisms resulting in the movement of genes from one population to another. Hence, it describes the exchange of genetic information between populations through migration. This flow, generally occurs within a species (Slatkin 1985). According to Balloux et.al., (2002) high gene flow precludes local adaptation (i.e. the fixation of alleles, which are favoured under local conditions), and will therefore also impede the process of speciation (Barton et.al.,1985). On the other hand, gene flow can generates new polymorphism in the populations, and increase local effective population size (the ability to resist random changes in allele frequencies), thereby opposing random genetic drift, generating new gene combinations on which selection can potentially act. Migration is sometimes used synonymously with gene flow, which is correct when the migration between established populations is the mechanism of gene flow. Therefore, the analysis of genetics population have been widely used for examining patterns of animal dispersal over both geographic and temporal dimension. Advances in population genetics theory and adoption of new types of molecular markers have been accompanied over decades, by parallel creation and improvement of analytical software programs to effectively calculate population genetics parameters. Although numerous software programs are available, only a few are used routinely in studies of natural population. Literature data have established that the most efficient measure to assess population structure is based on Wright's F-statistics (1951), Wright's inbreeding coefficient (F_{ST}) being particularly useful for analysing microsatellite markers because it is able to discriminate between alleles, especially that rare ones, although F_{ST} produced using such markers can sometimes be overestimates of the true value.

1.2 APPLICATION OF MOLECULAR MARKERS IN APHIDOLOGY

DNA markers has been used to obtain the “ first aphid genetic fingerprint” (Carvalho et.al.,1991). Few insects can match the ecological flexibility of aphids with their range of environmentally and genetically determined phenotypes (Williams and Dixon, 2007). They are therefore especially successful at exploiting diverse environmental conditions and host-plants adaptations. In the past 25 years or so, molecular markers revealed much new information about the biology of aphids. Some findings have been surprising, whilst others have only confirmed previous hypothesis, or have provided information of practical value for aphids control. Aphids are the “Russian dolls” of the animal world,

with the telescoping of generations within an individual accelerating their reproduction (Dixon, 1998, Harrington, 1994). Further other benefits include the purging of acquired genomic aberrations within annual parthenogenetic lines by selection, genetic drift and recombination. Some aspects of aphid biology that have greatly benefited from the application of molecular markers include phylogenetics, life cycle strategies, host plant adaptation and plant-mediated resistance, pathogenicity, insecticide resistance, dispersal and geographic range and geographic colonization (Normark et.al., 1999).

The greatest contribution of all markers in aphidology has been to enable characterization of genetic individuals within population (Harrington, et.al., 2007) revealing patterns of genetic variation in relation to life cycle (Hebert et.al., 1995), ecology, demography and climate (Hoy et.al., 2003). Not only, such markers facilitated phylogenetic differentiation (Morgan et.al., 2005) together with the understanding of important co-evolutionary associations of aphids with fungal entomopathogenes (Tyman et.al., 2004) and, bacterial symbionts (Simon et.al., 2003).

As already described, microsatellites are highly polymorphic compared with other classes of markers, and they have been used not only to answer several questions related to population genetics such as gene flow, but also for the study of the relationships between reproductive mode and ecological parameters. In this specific case, as described by Simon et.al., (1999a) in *Sitobion avenae*, a strong geographic partitioning of life cycle types across a north-south cline was revealed. On the other hand, microsatellites have been also used to reveal an apparent lack of recombination in the sex-determining chromosome in males and females using X-linked microsatellite loci in *Myzus antirrhinii* (snapdragon aphid), *S. avenae* and *Sitobion miscanthi* as a models (Hales et.al., 2002). Most of the studies concerning *Myzus persicae* regard the demographic distribution of its genotype in relation to life cycle and climate. Indeed, highly polymorphic markers have been used in various studies, notably by Fenton et.al. (2005) for Scottish populations, by Wilson et.al. (2002) for Australian populations and by Guillemaud et.al. (2003) for French populations. In the first works the authors analysed different DNA microsatellites in three dominant clones C, I and J, present in the Scottish populations of *Myzus persicae*. Hence, the purpose was to study the dynamics of insecticide resistant clones and their turnover from season to season, but also to identify other genotypes and their resistance to pesticides (Fenton et al., 2005). Wilson et.al., (2002) tested cross-species amplification of 48 microsatellite loci in species of the following genera: Aphidinae: Aphidini: *Aphis* and *Rhopalosiphum*; Aphidinae: Macrosiphini: *Acyrtosiphum*, *Brevicoryne*, *Diuraphis*, *Illinoia*, *Macrosiphoniella*, *Macrosiphum*, *Metopeurum*, *Metapolophium*, *Myzus*, *Phorodon*, *Sitobion* and *Uroleucon* and Neuenaphidinae: *Neuenaphis*. Their results show that cross-species application of known microsatellite loci is a highly promising source of codominant markers for population genetic and evolutionary studies in aphids. Finally,

in the latter study the authors examined the genetic variability at seven microsatellite loci of *M. persicae* populations in France, where both reproductive modes occur: continuous parthenogenesis and sexual reproduction once a year. The authors provide clear genetic evidence that the breeding system affects genotypic variability, as cyclically parthenogenetic aphids are far more variable than their obligately parthenogenetic counterparts. Finally, they observed that a temporal decrease in genetic variability and a temporal genetic differentiation effect suggest the existence of selective factors that play an important role in shaping the genetic structure of *M. persicae* populations.

Microsatellite analysis also allows to face one of the most important aspect of insect ecology, that is the study of aerial movement (Johnson 1969). Indeed, in aphids (Homoptera, Aphididae), there have been reports of extreme long-distance migrations, but it is likely that short distance dispersal is more common and a more important process for the ecology and genetic structure of aphid populations (Loxdale et.al., 1993).

1.2.1 Insecticide Resistance In *Myzus persicae*

All living species show genetic diversity that arises from naturally occurring mutations. When insecticides are used, natural selection will favor individuals more resistant to the insecticides. Pest crops may survive the treatment and reproduce to give a progressive build up of the particular genetic trait (insecticide resistance) in the population. This becomes apparent as control failures when these resistant insects start to dominate the population. *M. persicae*, for example, has developed a way of surviving exposure to insecticides: *M. persicae* females could reproduce without mating (parthenogenesis) so, large populations can build up quickly. The next generation of aphids carries the same genes as their mother, including any insecticide resistance traits. In some case, this form of reproduction could continue through the winter so carrying resistance forward to the following season.

At present, three different resistance mechanisms are known in *Myzus persicae*:

- **Esterase** - aphids with this resistance mechanism produce increased amounts of enzymes called esterases, which break down insecticides before they reach their target sites. This effect varies depending on the chemical structure of the insecticide. Resistance is greatest to organophosphorus (OP) insecticides although carbamates and pyrethroids are also affected to a lesser extent. *M. persicae* specimens can contain different amounts of esterase as a consequence of gene amplification (Cassanelli et. al., 2005).

- **Knockdown resistance or *kdr*** - pyrethroids act on another protein in the aphids nervous system called “sodium channel protein”, which is responsible for the passage of signals along the nerve. The insecticides hold these channels ‘open’ so that the nervous system becomes overexcited, leading to the death of the insects. *Kdr* resistant specimens contain a modified sodium channel protein, specifically insensitive to pyrethroids. The mechanism can be detected by a simple insecticide bioassay, although a DNA- based test is more suitable (Chen et al., 2010).

- More recently resistance to **neonicotinoid insecticides** of which imidacloprid was the first to be marketed (Jones et.al., 2011) has been described in *M. persicae* (Philippou et.al.,2010). The resistance in *Myzus persicae* was associated with reduced affinity of imidacloprid for the target site (the nicotinic acetylcholine receptor) as a result of mutation of a key amino acid residue (R81T) in the loop D region of a nAChR β 1 subunit (Puinean et.al., 2013).

M. persicae has historically been controlled with organophosphate, carbamate and pyrethroid insecticides (Contreras. et.al., 2013). More recently, neonicotinoids insecticides have been included in control programs, often used in combination with pyrethroids to enhance the aphids knock-down effect (Contreras et.al., 2007). Therefore there was an increasing use of neonicotinoids in particular on host crops of *M. persicae* (Puinean et.al., 2010). In many agricultural areas, *M. persicae* frequently is subjected to insecticide sprays on both primary and secondary host plants, which selects for insecticide resistance.

1.3 AIMS OF THE WORK

The objective of the present work will be to a deeper investigation on *Myzus persicae* populations structure and an assessment of the genetic variability in Italian populations of this species, within much of its range of distribution. For this purpose eight microsatellites were tested on large samples of aphids collected both directly from the field and others reared in the lab for a long time after their collection. The aim of the study was also to assess if the selection pressure was a unifying forces that could prevail over the diversifying forces as the geographical distance or genetic drift, especially in the field populations. Microsatellite markers were also used in order to examine if there were genotypes in linkage with host plant and/or collecting sites. In effect this species is extremely polyphagous (Blackman et.al., 2000), but there is also evidence of genetic variation with respect to host-plant adaptation. For example, in literature data (De Jong 1929) (Brain 1942) (Margaritopoulos et.al., 2007) it has been long suspected that

populations colonizing tobacco *Nicotiana tabacum* L. could be a different taxon from those on other crops.

Insecticide resistance is an example of a dynamic evolutionary process in which chance mutations conferring protection against insecticides are selected in treated populations (generally during the spring season). High levels of variability in color in *Myzus persicae*, its life cycle and insecticide resistance, reflect its ability to adapt as an agricultural pest (Foster 2000). However, it is known that genetic variability is generally low in asexually reproducing populations; in other words, few genotypes are spread over large geographic areas as described by Fuentes-Contreras et.al., (2013). For this reason both genetic drift and natural selection are often invoked to account for this low genetic variability. The peach-potato aphid, which include both cyclic and obbligate parthenogenesis, has developed several insecticide resistance mechanisms as a consequence of intense insecticide use since the 1950s. So, working with the field populations, in this PhD thesis it has been examined the genetic variability using both the profile of microsatellites and two different resistance genotypes (*kdr* and R81T). This study was necessary to determine whether the genetic structure in this species was driven by drift and/or selection. Data collected for this thesis tried also to understand if the selective pressures resulting from human activities, could modify considerably the genetic structure of Italian populations of *M. persicae*.

Finally, another key point of the study was to verify the presence of a linkage between microsatellites and specific resistance phenotypes (*kdr*-R81T) in order to identify a specific microsatellite marker able to trace some phenotypic trait associated with the resistance.

Moreover, focusing on the microsatellites variability and the resistance loci, the possible genotoxic effects of a pyrethroid pesticide (Brigade Flo) has been assessed *in vivo*, using a sub-lethal dose (LD70) in a clonal lineage with stable karyotype. A brief exposure time was chosen in order to validate a routine assay able to identify the genotoxicity and hence, to compete with the others biological system already described in literature (Bhunya et.al., 1988; Campana et.al., 2000) .

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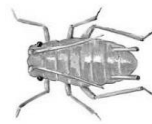
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2 MATERIAL AND METHODS

2.1 SAMPLING and DNA EXTRACTION

2.1.1 Field-Populations

The natural populations of *Myzus persicae* were composed of winged and wingless females adults and were mainly collected from peach (*Prunus persica* (L.) Batsch) as shown in Table 2.1.

Collecting Site	Region	Population	Lat/Long	Year	Host	N.of Individuals
San Prospero	Emilia-Romagna	Mp-109	44°21'52.40"N 11°46'42.35"E	2012	PEACH	10
San Martino in Fiume		Mp-113	44°10'48.16"N 12°14'34.90"E	2012	PEACH	10
Imola		Mp-127	44°22'11.99"N 11°44'44.00"E	2012	PEACH	10
Martorano		Mp-112	44°10'34.89"N 12°17'16.88"E	2012	PEACH	10
Pievesestina		Mp-102	44°11'10.52"N 12°13'21.54"E	2011	PEACH	8
CesenaA		Mp-125	44° 9'0.28"N 12°15'35.11"E	2012	PEACH	10
CesenaB		Mp-92	44° 7'53.47"N 12°15'32.58"E	2011	PEACH	4
Bagnacavallo		Mp-133	44°28'55.04"N 12° 1'19.56"E	2012	PEACH	9
Bologna		Mp-142	44°22'17.66"N 11°47'20.83"E	2012	PEACH	10
Roma	Lazio	Mp-155	41°38'38.41"N 12°39'58.47"E	2012	PEACH	10
San Salvo	Abruzzo	Mp-114	42° 2'24.96"N 14°44'17.76"E	2012	PEACH	10
Cerignola	Puglia	Mp-145	41°16'19.40"N 15°54'1.03"E	2012	PEACH	10

Table 2.1. List of the Italian *M. persicae* populations collected directly from the field

All aphids were collected directly from the plants in 2011-2012 during spring season. Crops were primarily treated with neo-nicotinoids and pyrethroids.

The samples come from different Italian regions, especially from various fields located in different Emilia-Romagna provinces, a few kilometres apart from each other, being located in an area of 3300 km² (Fig.2.2).



Fig 2.2. Map showing the collecting sites

2.1.2 Lab-Reared Clonal Populations

Further, during this study, 47 clonal lineages of *Myzus persicae* lab-reared for several years after the collection have been also analysed. The samples are listed in the Table 2.3 and as shown, most of the animals were collected from peach, but also from herbaceous plants like tobacco, tomato, potato and aubergine. All the populations were

reared on pea-seedlings (*Pisum sativum* cv ‘Meraviglia d’Italia’ under constant environmental conditions: 21°C, 16 h light:8 h dark photoperiod in order to maintain the parthenogenetic cycle as described in Blackman (1971).

2.1.3 DNA Isolation

For both types of populations, genomic DNA was obtained from single aphids (“whole-aphid DNA”) using a “salting-out” protocol (Sunnucks and Hales, 1996). Individual female wingless adults were crushed in a 1.5 mL microfuge tube and incubated at 55°C for 1h in 300 µl TNES (50 mM Tris, pH 7.5, 400 mM NaCl, 20 mM EDTA, 0.5% SDS) with 100 µl /mL Proteinase K. After 3-18 h, proteins were precipitated with 85 µl 5M NaCl and hard shaking for 15 sec. Proteins were pelleted in a microfuge at 14,000 rpm for 5 min. Then DNA was precipitated from the decanted supernatant with 1 volume 100% ethanol. DNA was pelleted, washed in 70% ethanol, air-dried, and dissolved in 20-50 µl sterile water in rough proportion to the size of the aphid. After quantification all DNA samples were diluted, if necessary, in sterile water in order to have a final concentration of 50ng/µl in order to perform a polymerase chain reaction (PCR). Regarding the 12 populations sampled directly from the field, for each sampled population 8 to 10 individuals were available, for a total of 111 extractions; while for the 47 laboratory reared populations 6 individuals were available for each population, for a total of 282 DNA extractions.

Collecting Site	Region	Population	Host	N.of Individuals
Torino1	Piemonte	Mp-53	PEACH	6
Torino2		Mp-46	PEACH	
Cuneo		Mp-14	PEACH	6
Lodi1	Lombardia	Mp-63	PEACH	6
Lodi2		Mp-84	PEACH	6
Como		Mp-83	PEACH	6
Verona1	Veneto	Mp-78	PEACH	6
Verona2		Mp-87	PEACH	6
Verona3		Mp-86	PEACH	6
Padova1		Mp-54H2	PEACH	6
Padova2		Mp-55	PEACH	6
San Prospero1	Emilia Romagna	Mp-93	PEACH	6
San Prospero2		Mp-95	PEACH	6
Bologna		Mp-3	PEACH	6
Ferrara1		Mp-37	PEACH	6
Argenta-Ferrara		Mp-12	PEACH	6

Ferrara2		Mp-7	PEACH	6
Imola1		Mp-45	PEACH	6
Imola2		Mp-68	PEACH	6
Imola3		Mp-59	PEACH	6
Imola4		Mp-24	PEACH	6
Ravenna1		Mp-27H	PEACH	6
Ravenna2		Mp-20	PEACH	6
Ravenna3		Mp-62	PEACH	6
Ravenna4		62H2	PEACH	6
Ravenna5		Mp-100H	PEACH	6
Piacenza1		Mp-69	AUBERGINE	6
Piacenza2		Mp-33H	PEACH	6
Piacenza3		Mp-31	TOMATO	6
Piacenza4		Mp-16	PEACH	6
Piacenza5		Mp-81	AUBERGINE	6
Piacenza6		Mp-17	PEACH	6
Pisa	Toscana	Mp-26	PEACH	6
Pesaro-Villanova	Marche	Mp-56	TOBACCO	6
Ascoli Piceno		Mp-58	PEACH	6
Chieti	Abruzzo	Mp-64H	TOBACCO	6
Colle Corvino		Mp-57	TOBACCO	6
Salerno	Campania	Mp-49	TOBACCO	6
Benevento		Mp-48	TOBACCO	6
Scafati		Mp-51	TOBACCO	6
Foggia	Puglia	Mp-76	PEACH	6
Cosenza	Calabria	Mp-70H1	PEACH	6
Lamezia1		Mp-34	PEACH	6
Lamezia2		Mp-23	POTATO	6
Castrovillari		Mp-22	PEACH	6
Catanzaro		Mp-90	PEACH	6
Cagliari	Sardegna	Mp-72	PEACH	6

Table 2.3. List of the lab-reared lineages of *M. persicae*.

2.2 MICROSATELLITE ANALYSIS

2.2.1 Molecular Markers Amplification

Each aphid was genotyped using eight different microsatellite loci: five (M35^A, M40^A, M49^A, M63^A, M86^X and M37^A) described in Sloane et al. (2001) and three (Myz2^A and Myz25^X) from in Wilson et al., (2002), all isolated from *M. persicae* (Table 2.4). Information on chromosomal location is incorporated into the locus name of each

species for which microsatellites have been cloned: A = autosomal, X = X-linked (Wilson et al., 2002). The microsatellites were amplified in a 10µl of final volume containing 2 units of GoTaq® DNA Polymerase (Promega), 25 mM MgCl₂, 0.2 µM dNTP, 0.5 mM of each primer and 1µl of the genomic aphid DNA.

Locus	Size range (bp)	Primer Sequences (5'-3')	Tm (C)	Reference
M40 ^A	123-135	F* : ACACGCATACAAGAATAGGG R : AGAGGAGGCAGAGGTGAAAC	55°	(Sloane et al., 2001)
M86 ^X	97-141	F* : TCCACTAAGACCTCAAACAC R : ATTTATTATGTCGTTCCGCC	54°	(Sloane et al., 2001)
Myz2 ^A	177-207	F* : TGGCGAGAGAGAAAGACCTGC R : TCGGAAGACAGAGACATCGAGA	58°	(Wilson et al., 2002)
Myz25 ^X	119-126	F* : AACCCATCTCACTCGTCAGCC R : GAATCTGGAGAGCGGTTAATGC	58°	(Wilson et al., 2002)
M35 ^A	178-198	F* : GGCAATAAAGATTAGCGATG R : TGTGTGTATAGATAGGATTGTG	55°	(Sloane et al., 2001)
M37 ^A	155-157	F* : GTGTGAGTAAGTCGTATTG R : TTGTATTATGTACCTGTGC	50°	(Sloane et al., 2001)
M49 ^A	130-199	F* : CCCATACATACCTCCAAGAC R : AGAGAGAAAATAGGTTCTGTG	55°	(Sloane et al., 2001)
M63 ^A	163-207	F* : GCGGTTTTCTTTGTATTTTCG R : GATTATGGTGCTCGGTGG	55°	(Sloane et al., 2001)

Table 2.4. Details of *Myzus persicae* microsatellites used in this study. Asterisks denote primers marked with fluorescent dye.

PCR reactions were performed in a T-personal thermocycler (Biometra, Goettingen, Germany) as follows: initial denaturation at 95°C for 10 min followed by 30 cycles of denaturation at 95°C for 30s , annealing temperature depending on the locus (See Table 4) and elongation at 72°C, each for 30s. 4 µl of amplification products were then loaded onto a 2% Agarose Gel (Sigma-Aldrich) using peqGold 50 bp DNA-Ladder (Biotechnologie GmbH) as a molecular marker. The forward primer of each microsatellite locus was labelled at the 5' end with a fluorophore 6-carboxyfluorescein (6-FAM) which is the most commonly used fluorescent dye for labelling oligonucleotides (provided by Sigma-Aldrich).

2.2.2 Data Processing Tool by Automatic Sequencer and GeneMapper Software

The capillary electrophoresis necessary to analysed the fragments, was performed with the Applied Biosystems® 3130 Genetic Analyzer 4-capillary instrument kindly offered by Professor Milo Frattini at the Laboratory of Clinical Pathology and Molecular Diagnostics of Istituto Cantonale di Locarno, Switzerland (Fig.2.5).

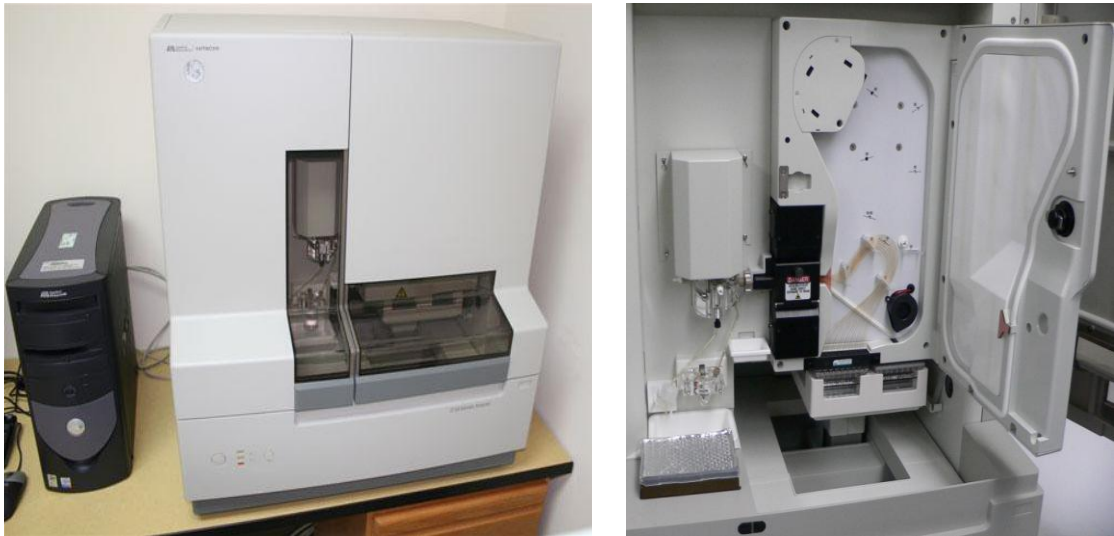


Fig. 2.5. Applied BioSystems 3130 Instrument (from Lab Instrument Provider Garden State Scientific)

1 μ l of product was mixed in 10 μ l of deionized formamide and 0.5 μ l of GeneScan-500 LIZ size standard (Fig.2.6), which allows the determination of the size of the DNA fragments with a length range 35 and 500 nucleotides. Subsequently, the samples were denatured at 95°C for 3 minutes, added on the plate and then loaded on the instrument.

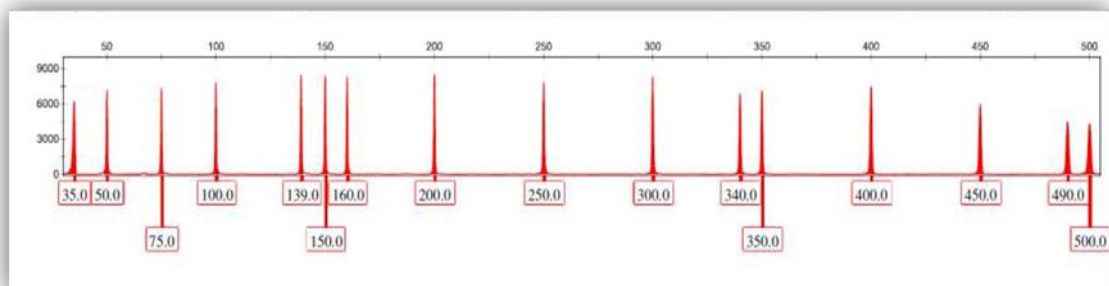


Fig. 2.6. GeneScan-500 LIZ size standard (Image Adapted from the Manual of the Genetic Analyzer 3130, Applied BioSystems)

During the denaturation process the double strands were separated and thus migrate at different speeds when subjected to capillary electrophoresis. The size resolution has to be good enough to distinguish between fragments varying in length by as few as two bases. To achieve this, a size standard “ladder” has to be run concurrently in each capillary to create a standard curve of sufficient precision. The size standard should be labelled with a different coloured fluorescent dye from the analysed fragments. The data collected in electrophoresis were elaborated using GeneMapper® software (Fig.2.7) allowing for the genotyping of aphids. Indeed, this specific program permit a visual inspection of each amplified fragment for the specific microsatellite locus. Each fragment was showed in a graph: the X-axis has the time of the migration on gel and the Y-axis the intensity of the amplified product. So, using these parameters each allelic peak has allowed to analyse the genotype of each single aphid. The results of the length of alleles were computed in base pair (bp),each data set consisted of a whole number with two decimal places, hence the need to round the data.

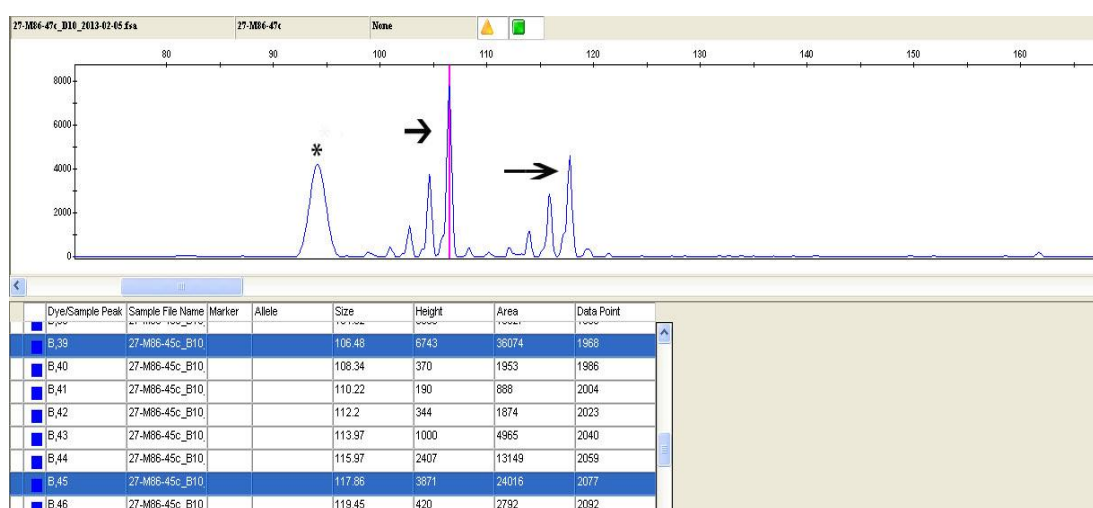


Fig. 2.7. GeneMapper® software data analysis produces intuitive data tables for fast data review. The main peaks, highlighted with black arrows, are the alleles. Ideally, microsatellite data produce only two bands but often there are minor peaks in addition to the major ones: they are called stutter peaks and they usually differ from the major peaks by two nucleotides. Asterisk shows an artefact.

As shown in the figure 2.7 by GeneMapper it is also possible to recognize amplification chemistry artefacts, including Plus A and stutter peaks, by differentiating among microsatellite repeats of varying lengths and produces tables that can be sorted in a format that is easy to interpret. Finally, *GeneMapper* algorithm represents the best system for its extreme sensitivity, specificity and accuracy.

2.2.3 Sequencing Of M49^A Locus

The locus M49 showed abnormal peaks in electropherograms, as highlighted in Fig. 2.8. Therefore, the microsatellite products were sequenced in order to identify the flanking regions and to check if the two alleles had exactly the same flanking regions including, of course, the primer site/s. After amplification the products were run on a 2% electrophoresis gel and the specific bands were cut and purified using the High pure PCR product purification- Kit (Roche). Successively a sequencing reaction was performed using 2 µl of purified DNA, TRR mix 0,5 X, Sequencing Buffer 0,5X and primer R only (without labelling, 0,16 µM), following this thermic profile: initial denaturation at 96°C for 1 min, followed by 25 cycles of 96°C for 10 min, 50°C for 5 min and 60°C for 4 min. The products were then purified using the Post CycleSeq purification G50 Dye Terminator Removal Kit (Real Biotech.). Finally, 1.5 µl of the samples were added to 15µl of formamide at 95°C for 3 min and plated.

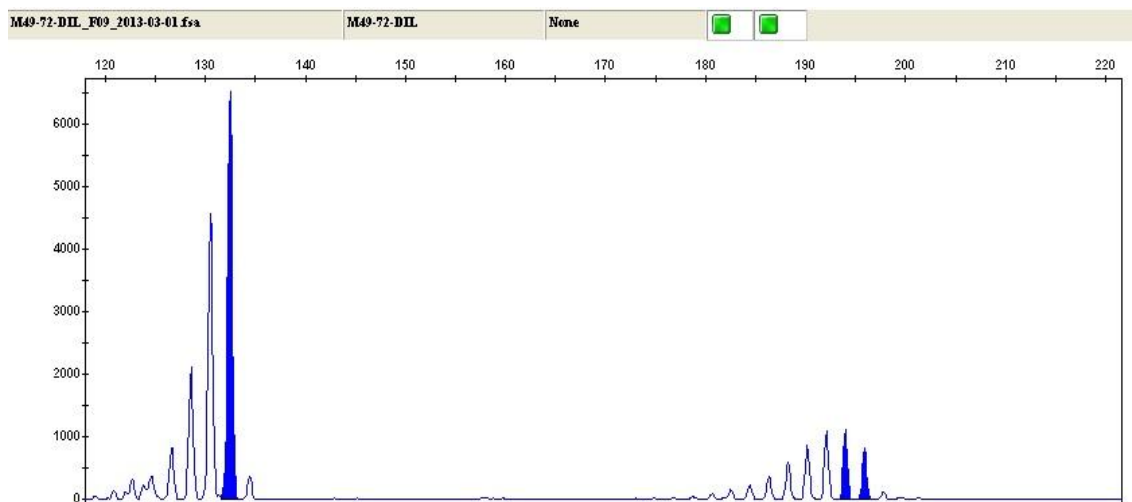


Fig. 2.8. Electropherogram evidencing abnormal peaks (in blue) of the M49 microsatellite

2.3 STATISTICAL ANALYSIS OF GENETIC DATA

2.3.1 *Microsatellite Variability: Allele Frequencies and Hardy-Weinberg Equilibrium*

Population genetics is the study of the distribution of and change in allele frequencies under the influence of the four evolutionary forces: natural selection, genetic drift, mutation, and migration. The descriptive statistics are used to summarize a collection of population data in a clear and concise manner (Barbujani et.al., 1997). A crucial concept in population genetics is the Hardy-Weinberg principle, which predicts how gene frequencies will be inherited from generation to generation given a specific set of assumptions (Thompson et.al., 1991). The Hardy-Weinberg principle states that in a large randomly breeding population, allelic frequencies will remain the same from generation to generation assuming that there is no mutation, gene migration, selection or genetic drift (Tishkoff et.al., 2004). This principle is important because it gives a standard from which to measure changes in allele frequency in a population.

In order to evaluate the genetic variability in Italian population of *Myzus persicae*, eight microsatellite loci were screened for each specimen in all population. The software GENALEX 6.41 (Peakall et.al., 2006) was utilized to obtain: (i) the number of alleles per locus, (ii) allele frequencies, (iii) probability to fit to Hardy–Weinberg equilibrium (HWE) using the dual null hypothesis of both heterozygote excess and deficit at individual loci, as well as over all loci, (iv) percentage of polymorphic loci for each population and (v) finally this program is able to identify the private alleles by population.

Observed and expected heterozygosity were computed using GENETIX 4.05 (Belkhir et.al., 2004), whilst allelic richness (A_c) was calculated through FSTAT 2.9.3 program (Goudet 2001). Given that polymorphism at annealing sites of the microsatellite primers can prevent the amplification of a particular allele, therefore resulting in heterozygote deficiencies, the presence of null alleles was tested with MICROCHECKER 2.2.1 (Van Oosterhout et.al., 2004).

2.3.2 *Genetic Analysis Among Populations*

Differences in the genetic structure between samples and among sample groups analyzed by testing for genetic and genotypic differentiation (test described in Raymond and Rousset, 1995b) and by calculating multilocus F_{ST} values (Weir et.al., 1984) were computed using FSTAT 2.9.3 (Goudet 2001). Wright's F-statistics (1951), is the most efficient measure to assess population structure: Wright's inbreeding coefficient (F_{ST} , also called θ) being particularly useful for analyze microsatellite markers because it is

able to discriminate between alleles, especially that rare ones, although F_{ST} obtained using such markers can sometimes overestimate the true value (Oliveira et.al., 2006). The fixation indexes F_{IS} , F_{IT} and F_{ST} , introduced by Wright in the early 1950s offer a convenient way to represent the structure of a population and its division into sub-populations. The excess of homozygosity (index of subdivision of populations and/or inbreeding) is commonly studied by applying the F statistics, defined by Wright (1978) and revisited by Weir and Cockerham (1984) in terms of analysis of variance. According to Wright, populations possess two levels of complexity , which can be estimated by two indices:

- F_{IS} , which measures the consanguinity (inbreeding) relative of individuals (I) compared to the population (S) to which they belong .
- F_{ST} , which measures the fixation of an allele due to random genetic drift, in terms of reduced heterozygosity of the population (S) with respect to the total population (T). This index is used to estimate the distribution of the populations (S).

In this way, the genetic variability can be divided into a component intra-populations (F_{IS}) and another component between populations (F_{ST}). The division of populations to drift and inbreeding are partly overlapping phenomena that cause both a deviation from the expected genotype frequencies under HWE toward an excess of homozygotes. Hence, population structure was assessed by calculating multilocus F_{ST} values for pairwise comparison of samples using GenALEX version 6.5.

Using the F_{ST} statistics it is possible to quantify the gene flow. Gene flow is fundamental for the maintenance of populations because it allows genetic diversity to be maintained by acting directly on the population structure and against random genetic drift. Thus gene flow results in homogenization of allelic frequencies and it has exactly the opposite effect of genetic drift, which tends to make populations genetically more heterogeneous (Avise et.al., 1994). The number of migrants per generation (N_m), where (N) is the population size and m is the proportion of migrants was assessed using GENETIX 4.05.

An assignment test (Ass) was also performed in order to evaluate the probability for each genotype/individual in each population to occur in a different one. The test was computed with GENECLASS2 (Piry et.al., 2004) based on Paetkau et.al., (1995) algorithm.

A Mantel Test was performed using GENALEX 6.41 (Peakall et.al., 2006). This test is useful because it allows to assess the correlation between the genetic distance (or similarity) matrix calculated with F_{ST} with the matrix of geographical distances (Bohonak 2002). The geographical coordinates of sampling sites were determined using Google Earth ® (2009).

Furthermore, Nei's standard genetic distance was calculated (Nei et.al., 1983) by using GENETIX 4.05 and a Neighbor Joining tree was computed using POPULATIONS

1.2.28. (written by Olivier Langella). Nei distance assumes that genetic differences arise due to mutation and genetic drift. It has the property that, if the rate of genetic change does not vary between loci, Nei's standard genetic distance represents the number of changes per locus.

Finally, the analysis of genotypic linkage disequilibrium (LD) between each pair of loci in each sample, including also the resistance loci *kdr* and R81, was carried out with GENEPOP version 4.0 (Raymond et.al., 1995). LD parameter refers to the tendency for two “alleles” to be present on the same chromosomes (positive LD), or not to segregate together (negative LD). Hence, linkage disequilibrium is the occurrence of some combinations of alleles or genetic markers in a population more often or less often than would be expected from a random formation of haplotypes from alleles based on their frequencies (Reich 2001).

2.4 *IN VIVO* GENOTOXICITY OF THE PYRETHROID *Brigade Flo* PESTICIDE

Pyrethroid resistance is correlated with the presence of the *kdr* mutation (i.e. the presence of a T instead of C at position 322 of the para-like gene. Sodium channel proteins (at the basis of *kdr* resistance are located on aphids nervous tissues. In order to assess the genotoxic effects of *Brigade Flo*, a synthetic pyrethroid insecticide (the most commercialised insecticide in agricultural field) *M. persicae* specimens have been treated with 10µg/mL of active principle (corresponding to a sub-lethal dose LD70), that while having a minimum of selective pressure, it allows the survival of a number of individuals. The LD dosage is normally the starting dose for the selection of resistance in the laboratory when starting from heterogeneous populations. Moreover, the doses were defined following a dose - response mortality curve, which was previously constructed at the laboratory of the “ Institute of Entomology and Plant Pathology ” Catholic Universita’ del Sacro Cuore in Piacenza, whereby this part of the study has been carried out. Hence, the genotoxicity has been studied *in vivo*, in a clonal lineages of *M. persicae* called 4H1 strain which showed a stable karyotype $2n = 12$. This clonal population was preferred to field populations because, in this last case, it could be difficult to identified the “native” variability from the variability induced by genotoxic effect. The DNA was obtained by single aphids as described in Guillemand et.al., (2011) and the aphids were screened for genotoxicity experiment using 8 microsatellites Myz2^A, Myz25^X, M35^A, M40^A, M49^A, M63^A, M86^X and M37^A.

Twenty wild-type aphids were used as a control, while 30 adult female aphids were moved in each pea plant, in order to begin the process of infestation. The plants were infested one week after the shoot was sufficient high in order to support the aphids. The day after, when the aphids were on the plants, aphids and plants were treated with

insecticide using specific LD70 dosages. In order to evaluate the genotoxicity effect, three total treatments have been carried out. Each treatment has been performed after 7 days from the previous one. After all, 10 animals for each treatment have been used for microsatellite analysis, following the protocol described in paragraph 2.2.



3 RESULTS

3.1 ALLELES ANALYSIS

3.1.1 Determination Of Alleles Size In Field-Populations By Capillary Electrophoresis

The first analysis regards twelve populations of *M. persicae* sampled directly from the field. Each population was composed of 8 to 10 individuals. A total of 111 individuals were examined. The analysis of each single individual of the different populations showed a substantial difference in genotypes over all loci. All results are reported in the final section relative to the supplementary data (Table A). The alleles size-range, analyzed through Genetic Analyzer 4-capillary instrument are represented in Fig.3.1.

Locus	Size range (bp)	Motifs	Tm (C)	Polymorphic/Monomorphic
M40 ^A	118-132	(AC)17	55°	P
M86 ^x	93-143	(CA)23	54°	P
Myz2 ^A	176-206	Unknown	58°	P
Myz25 ^x	116-142	Unknown	58°	P
M35 ^A	171-195	(AT)9(AC)22	55°	P
M37 ^A	152-160	(AC)16	50°	P
M49 ^A	130-204	(AC)31	55°	P
M63 ^A	167-195	(AC)29	55°	P

Fig 3.1. Alleles' size after capillary electrophoresis. Motifs of loci Myz2^A and Myz25^x are not determined because their sequence has not been reported in the original paper (Sloane et.al., 2001), even though they are indicated as di-nucleotidic repeats.

Moreover, all 8 loci were found to be polymorphic and therefore suitable for population analysis. As described in chapter 2.2.3, the M49 microsatellite showed significant abnormal peaks in some populations collected from the field but also in populations lab-reared for some years.

First of all the PCR conditions were optimized in order to exclude some technical errors but also the possibility that abnormal peaks were artefacts due to the Taq polymerase ‘slippage’ during the replication of the allele.

After this, the direct M49 satellite has been sequenced both in the specimens that showed abnormal peaks and in the specimens without abnormal peaks. The result showed that the flanking regions appeared to be the same after comparison with normal sequence as highlight in figure 3.2.

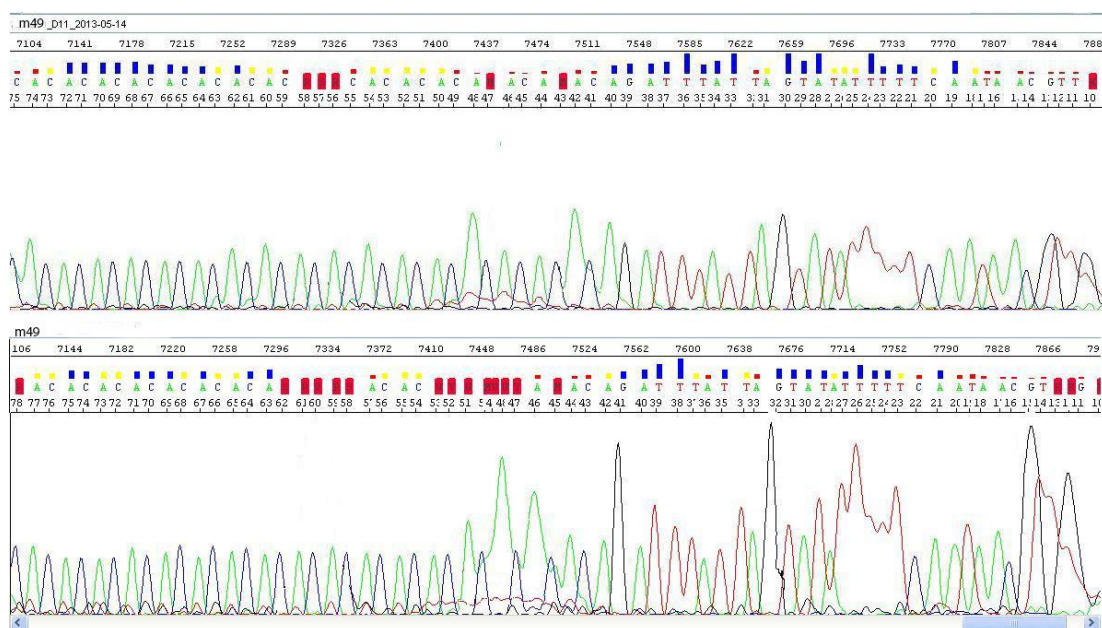


Fig.3.2. Example of sequences obtained for M49 locus.

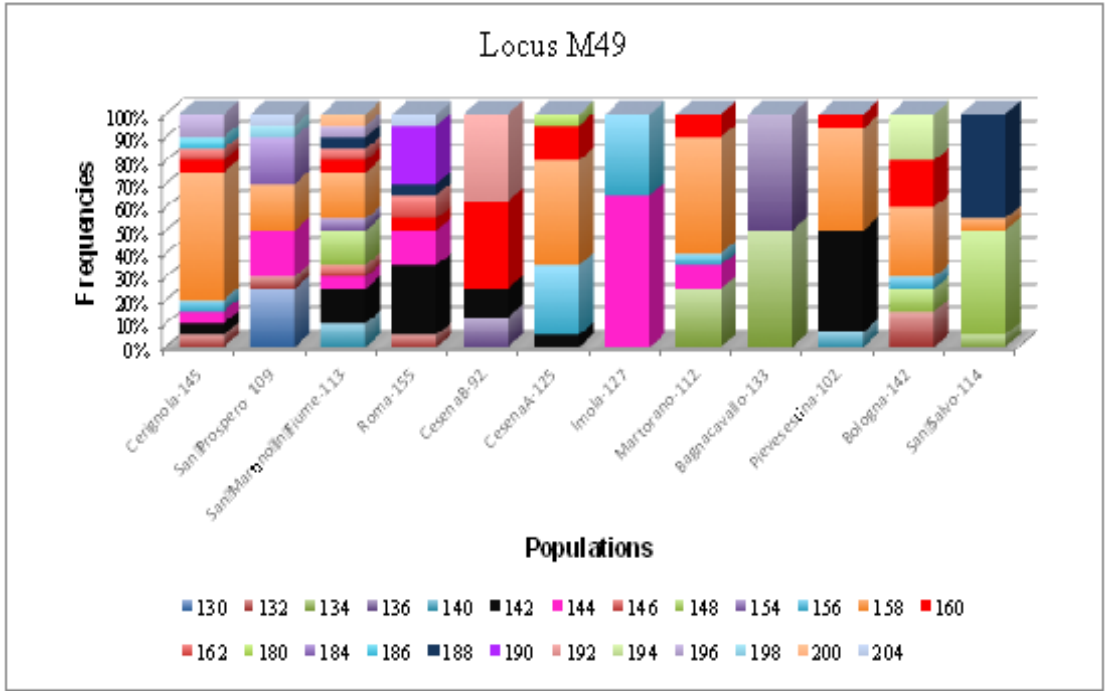
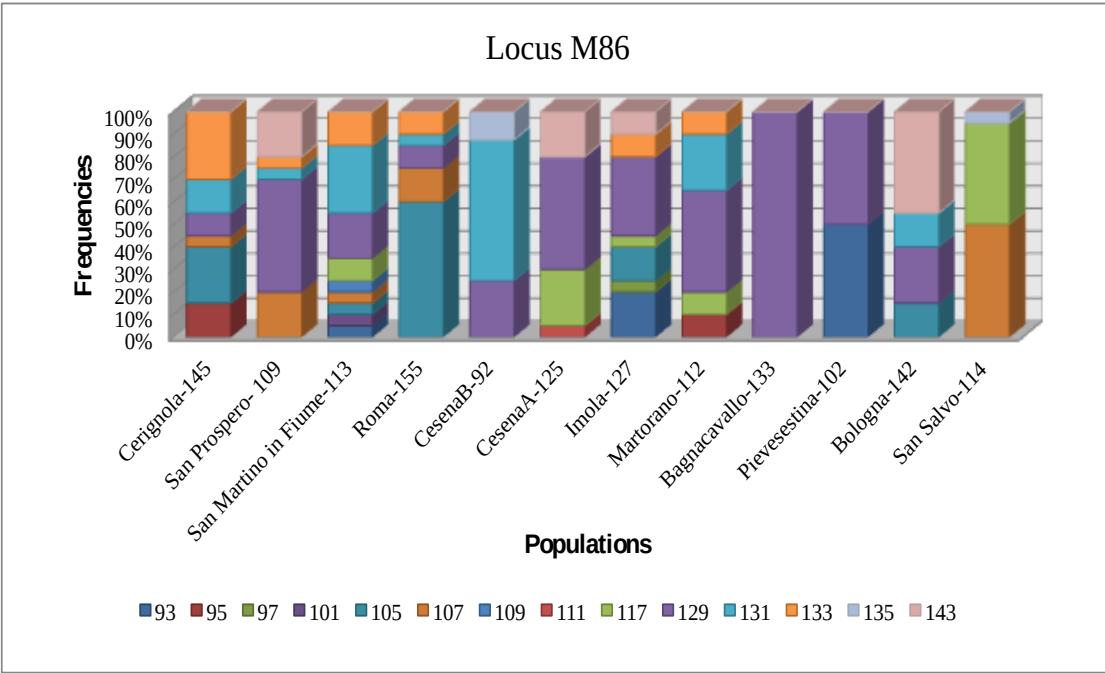
3.1.2 Genetic Diversity Within Field-Populations

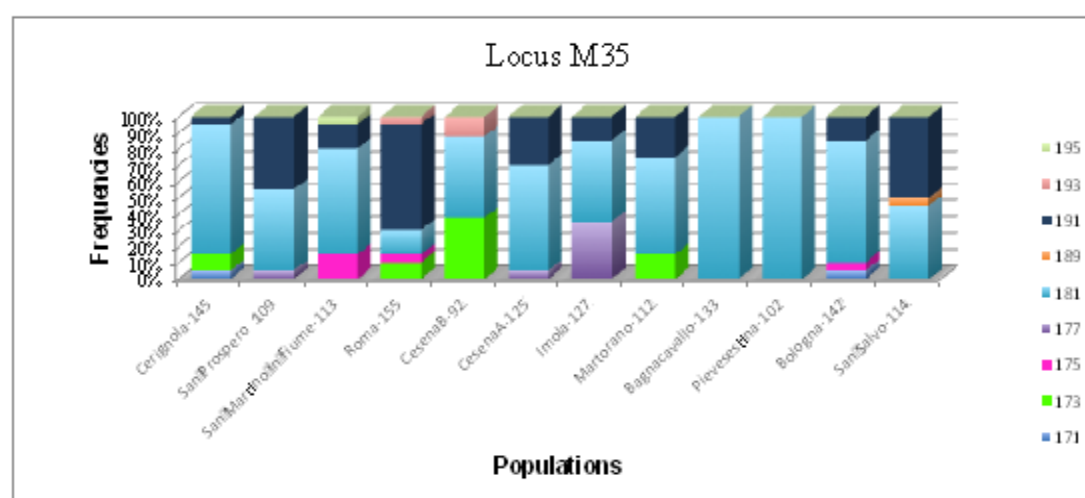
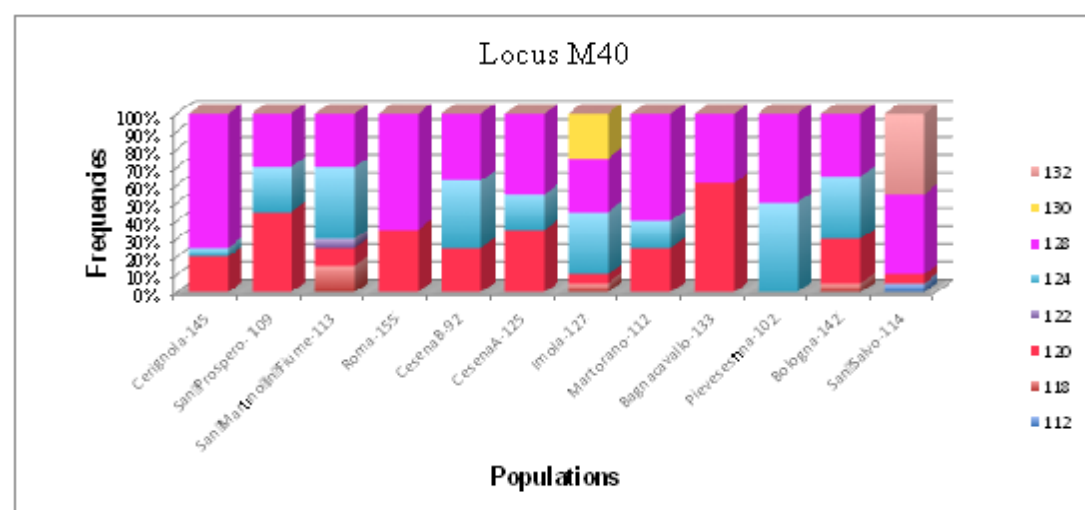
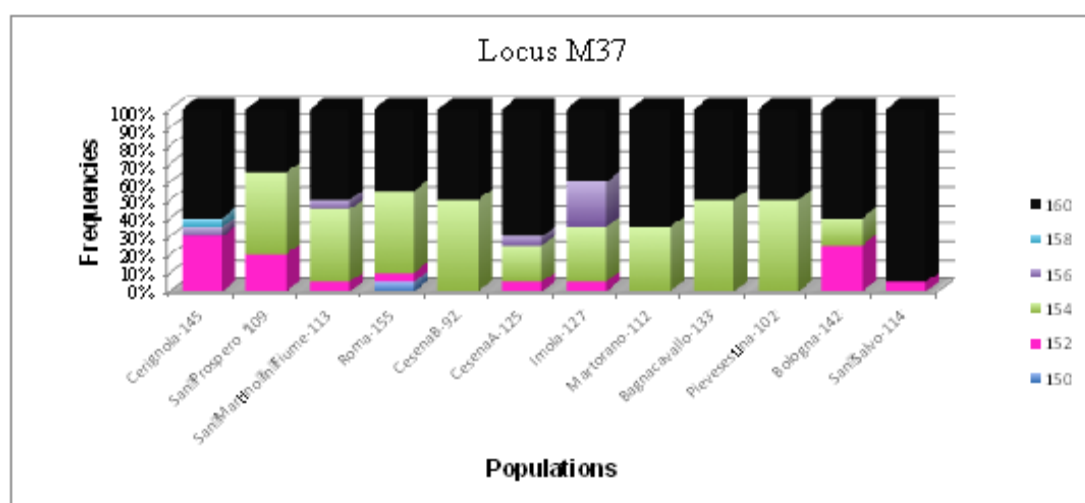
A substantial intra-population genetic variation was observed. A total of 90 alleles were identified for all 8 microsatellites. All loci analysed were polymorphic, but not for all populations (Fig.3.3). The observed exceptions were only for a few loci: Myz2 and Myz25 for the sample Pievesestina-102 and M63, Myz2 and M35 for the population Bagnacavallo-133, which were monomorphic.

Population	%P
Cerignola-145	100,00%
San Prospero-109	100,00%
San Martino in Fiume-113	100,00%
Roma-155	100,00%
Cesena B-92	100,00%
Cesena A-125	100,00%
Imola-127	100,00%
Martorano-112	100,00%
Bagnacavallo-133	50,00%
Pievesestina-102	62,50%
Bologna-142	100,00%
San Salvo-114	100,00%
Mean	92,71%
SE	4,98%

Fig.3.3. Percentage of Polymorphic Loci

Figs.3.4 show the allele frequencies per locus for each population. This histogram representation illustrates more clearly the different degree of polymorphism across all loci. M86 and M49 result the most polymorphic, showing a higher allelic variability within the majority of the study populations. Exclusively for the loci M37, M40, M35, Myz2, Myz25 and M63 all populations shared a most common allele as shown in bracket: M37(160), M40(128), M35(181), Myz2(200), Myz25(116) and M63(171). Allelic richness (AC), calculated per each locus and populations was between 2.812 to 5.969 (Table 3.6).





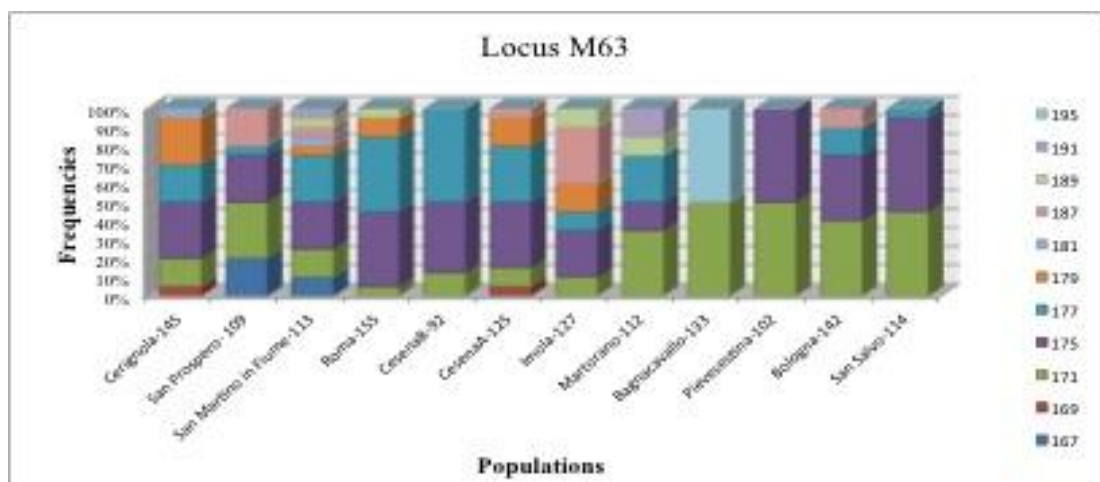
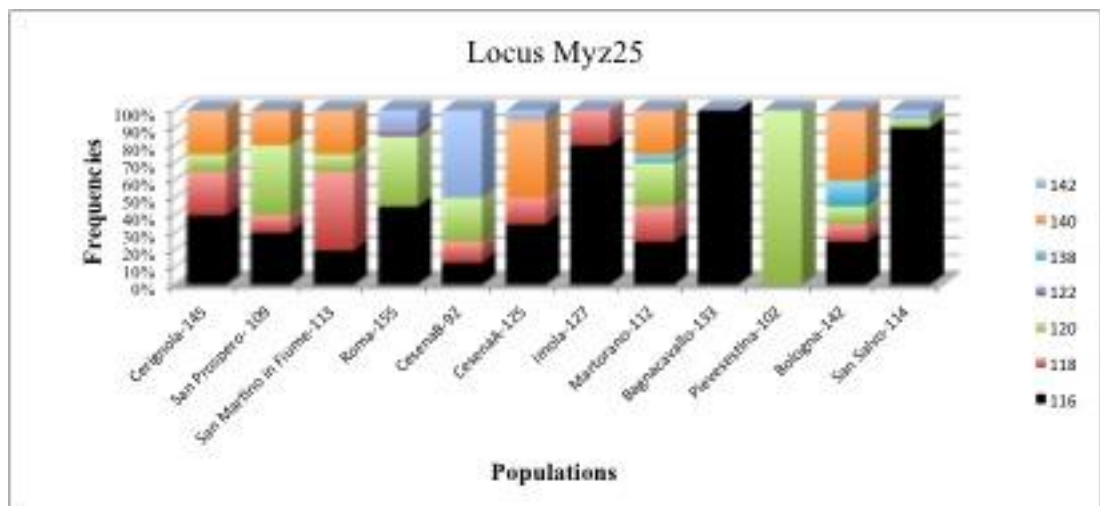
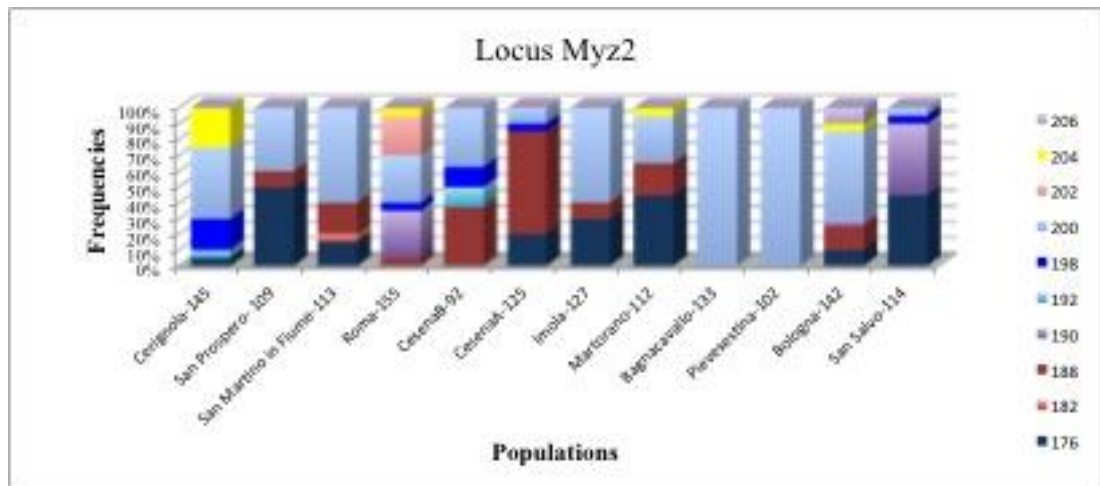


Fig 3.4. Allelic frequencies for the loci in the analysed populations. The first two histograms are relate at the two most polymorphic loci (M49-M86).

Considering all the 90 alleles identified during the sequencing, 28 were ‘private’ alleles, i.e. they were found only in a specific population. Among the latter, 18 (64%) had frequencies less than 0.100, resulting as ‘rare’ alleles.

The outcome of the frequency of the private alleles by these populations: San Salvo-114 and Bagnacavallo-133 is very high, indeed the value ranging respectively from 0.450 for the allele 132 in locus M40 and 0.500 for allele 195 in locus M63 (Fig 3.5).

Summary of Private Alleles by Population			
Populations	Locus	Allele	Freq
San Prospero- 109	M49	130	0,250
San Prospero- 109	M49	184	0,200
San Prospero- 109	M49	198	0,050
San Martino in Fiume-113	M86	101	0,050
San Martino in Fiume-113	M86	109	0,050
San Martino in Fiume-113	M35	195	0,050
San Martino in Fiume-113	Myz2	182	0,050
San Martino in Fiume-113	M40	122	0,050
San Martino in Fiume-113	M49	146	0,050
San Martino in Fiume-113	M49	154	0,050
San Martino in Fiume-113	M49	200	0,050
San Salvo-114	M35	189	0,050
San Salvo-114	M40	112	0,050
San Salvo-114	M40	132	0,450
CesenaA-125	M86	111	0,050
CesenaA-125	M49	180	0,050
CesenaA-125	M86	97	0,050
CesenaA-125	M40	130	0,250
Bagnacavallo-133	M63	195	0,500
Bologna-142	Myz2	206	0,100
Bologna-142	M49	194	0,200
Cerignola-145	M49	186	0,050
Cerignola-145	M37	158	0,050
Roma-155	Myz2	202	0,250
Roma-155	Myz25	122	0,050
Roma-155	M49	190	0,250
Roma-155	M37	150	0,050
CesenaB-92	M49	192	0,375

Fig 3.5. Private alleles in field-populations

Table 3.6. Allelic Richness (Ac), observed (Ho)/expected (He) heterozygosity, null alleles (Null) and Hardy-Weinberg Equilibrium (HWE) per locus and population. F-statistics between populations (F_{ST}) and within populations (F_{IS}). Asterisks show data significance (*= $P<0,05$; **= $P<0,01$; ***= $P<0,001$).

Populations	Statistical Analysis	Loci								Over All Loci for each Pops
		M86	M63	M35	Mys2	Mys25	M40	M49	M37	
Cerignola-145	Ac	4.592	4.430	2.453	3.645	3.547	2.298	4.453	2.776	
	H_o/H_e	0,800/0,790	0,800/0,780	0,400/0,345	0,600/0,690	0,800/0,705	0,500/0,395	0,600/0,670	0,800/0,545	0,663/0,615 (ns)
	Null	No	No	No	No	No	No	No	No	
	HWE	ns	ns	ns	***	ns	ns	ns	ns	
San Prospero- 109	Ac	3.595	4.121	2.398	2.648	3.523	2.924	4.842	2.886	
	H_o/H_e	0,600/0,665	1,000/0,765	0,100/0,545	0,200/0,580	0,800/0,700	0,600/0,645	1,000/0,810	0,500/0,635	0,600/0,668 (ns)
	Null	No	No	Yes	Yes	No	No	No	No	
	HWE	*	**	*	*	**	**	***	*	
San Martino in Fiume-113	Ac	5.334	5.357	3.014	3.105	3.498	3.832	6.364	2.796	
	H_o/H_e	1,000/0,825	1,000/0,830	0,600/0,530	0,500/0,575	0,600/0,685	0,900/0,715	0,900/0,885	1,000/0,585	0,813/0,704 (ns)
	Null	No	No	No	No	No	No	No	No	
	HWE	ns	ns	ns	ns	ns	ns	ns	ns	
Roma-155	Ac	3.512	3.445	3.260	4.101	3.047	1.990	4.985	2.797	
	H_o/H_e	0,400/0,595	1,000/0,665	0,400/0,540	0,800/0,750	0,800/0,625	0,700/0,455	1,000/0,805	0,900/0,590	0,750/0,628 (ns)
	Null	No	No	No	No	No	No	No	No	
	HWE	*	*	ns	**	*	ns	ns	ns	
CesenaB-92	Ac	3.000	3.000	3.000	4.000	4.000	3.000	4.000	2.000	
	H_o/H_e	0,250/0,531	1,000/0,594	1,000/0,594	1,000/0,688	0,750/0,656	1,000/0,656	1,000/0,688	0,000/0,500	0,750/0,613 (ns)
	Null	No	No	No	No	No	No	No	No	
	HWE	ns	ns	ns	ns	ns	ns	ns	*	
CesenaA-125	Ac	3.246	4.226	2.376	2.950	3.195	2.886	3.582	2.698	
	H_o/H_e	1,000/0,645	0,900/0,750	0,600/0,485	0,500/0,525	1,000/0,650	1,000/0,635	0,800/0,680	0,500/0,465	0,788/0,604 (ns)
	Null	No	No	No	No	No	No	No	No	
	HWE	ns	ns	ns	ns	ns	ns	ns	ns	
Imola-127	Ac	4.800	4.690	2.796	2.629	1.898	3.715	1.990	3.321	
	H_o/H_e	1,000/0,790	1,000/0,795	1,000/0,605	0,200/0,540	0,400/0,320	0,800/0,720	0,700/0,455	0,800/0,685	0,738/0,614 (ns)
	Null	No	No	No	Yes	No	No	No	No	
	HWE	***	*	*	*	ns	***	ns	ns	
Martorano-112	Ac	3.906	4.205	2.756	3.273	4.145	2.756	3.654	1.990	
	H_o/H_e	0,900/0,705	1,000/0,760	0,500/0,555	1,000/0,665	0,800/0,770	0,800/0,555	0,800/0,665	0,700/0,455	0,813/0,641 (ns)
	Null	No	No	No	No	No	No	No	No	
	HWE	ns	ns	*	ns	ns	ns	**	ns	
Bagnacavallo-133	Ac	1.000	2.000	1.000	1.000	1.000	1.996	2.000	2.000	
	H_o/H_e	0,000/0,000	1,000/0,500	0,000/0,000	0,000/0,000	0,000/0,000	0,778/0,475	1,000/0,500	1,000/0,500	0,472/0,247 (ns)
	Null	No	No	No	No	No	No	No	No	
	HWE	Monomorphic	**	Monomorphic	Monomorphic	Monomorphic	ns	**	**	
Pievesestina-102	Ac	2.000	2.000	1.000	1.000	1.000	2.000	2.999	2.000	
	H_o/H_e	1,000/0,500	1,000/0,500	0,000/0,000	0,000/0,000	0,000/0,000	1,000/0,500	1,000/0,609	1,000/0,500	0,625/0,326 (ns)
	Null	No	No	No	No	No	No	No	No	
	HWE	**	**	Monomorphic	Monomorphic	Monomorphic	**	***	**	
Bologna-142	Ac	3.562	3.446	2.607	3.912	4.057	3.328	4.631	2.756	
	H_o/H_e	0,800/0,690	0,600/0,685	0,500/0,410	0,800/0,650	0,900/0,735	0,700/0,690	1,000/0,795	0,600/0,555	0,738/0,651 (ns)
	Null	No	No	No	No	No	No	No	No	
	HWE	ns	ns	ns	ns	ns	ns	ns	ns	
San Salvo-114	Ac	2.398	2.398	2.398	2.797	1.800	2.797	2.797	1.400	
	H_o/H_e	1,000/0,545	1,000/0,545	1,000/0,545	1,000/0,590	0,100/0,185	1,000/0,590	1,000/0,590	0,100/0,095	0,775/0,461 (ns)
	Null	No	No	No	No	No	No	No	No	
	HWE	*	*	*	***	***	***	***	ns	
Over All pops each Locus	Ac	4.960	4.446	2.873	3.866	3.728	3.435	5.969	2.812	
F-Statistic All Pops	F_{IS}	-0,202	-0,383	-0,184	-0,056	-0,152	-0,391	-0,325	-0,293	
	F_{ST}	0,262	0,139	0,219	0,263	0,314	0,148	0,248	0,122	

To test for deviation from random mating, the ‘exact Hardy–Weinberg test’ of Guo & Thompson (1992) was applied separately to each locus. The calculations for Hardy-Weinberg equilibrium (HWE) was also necessary in order to measure how selection and mutations could cause changes in the allele frequencies of a gene pool over time including a comprehensive example of the evolution of pesticide resistance. The instances of HWE departure were identified across all loci in two populations: Pievesestina-102 and San Prospero-109 (see Table 3.6). However, a few cases of multilocus significant deviations from Hardy-Weinberg were observed mainly for seven populations: Cerignola-145 (Myz2), Roma-155 (M86, M63, Myz2, Myz25), Cesena B-92 (M37), Imola-127 (M86, M63, M35, Myz2, M40), Martorano-112 (M35, M49), Bagnacavallo-133 (M63, M49, M37) and San Salvo-114 (M86, M63, Myz2, Myz25, M49, M40, M35). The analyses for the presence of null alleles suggested their presence for Myz2 and M35 loci only for two populations (San Prospero-109 and Imola-127) that also exhibited deviation from HWE (Table 3.6). Sometimes the presence of null alleles probably could be the reason of the deviation from HWE. Only 3 populations out of 12 tested, were in HWE: San Martino in Fiume-113, CesenaA-125 and Bologna-142. As null alleles were suspected only for very few loci in only two populations, I performed the analysis taking these loci into account.

Observed heterozygosity (H_o) over all loci ranged from 0.500 to 1.000. The absence of correlation between number of analysed individuals and observed heterozygosity could suggest that the scored variability is not linked to sample size. The analyses identified a higher number of observed heterozygous genotypes (H_o is higher than H_e) in the following populations: San Prospero-109, Pievesestina-102, Martorano-112, San Salvo-114, San Prospero-127, Bagnacavallo-133 and Roma-155 as shown in Table (3.6). Therefore, these data could support the deviation from HWE of these populations listed above, suggesting the probability that these populations could have a higher fitness which could help to increase their variability or also, these species could keep unchanged their specific phenotype for a particular genotype (microsatellite locus).

3.1.3 Genetic Differentiation Between Field- Populations

Wright’s F statistics (e.g. F_{ST} and F_{IS}) are fundamental for estimating the effective populations size of samples, but also to describe the heterozygosity levels in a population. The reduction in observed heterozygosity (increased homozygosity) could be caused by inbreeding and natural selection. This measure can be used to quantify the level of genetic differentiation between the subpopulations. This quantification

has been formalised in the first instance by Wright (1968,1978, 1990) in a series of hierarchical F-statistics:

F_{IS} : inbreeding coefficient;

F_{ST} : measures the effect of population subdivision, which is the reduction heterozygosity in a subpopulation due to genetic drift (Crow et.al., 1984);

The rate of inbreeding F_{IS} was estimated for all populations for all loci (Table 3.6). In the samples analysed, almost all F_{IS} indices are negative with a mean value -0.265 ± 0.043 , indicating an heterozygotes excess (outbreeding). This data could suggest a possible mixing of populations that contribute to the genetic variability.

Instead the overall F_{ST} significant value indicates a substantial genetic differentiation and therefore a substantial structuring; these data are corroborated by the average calculated among all F_{ST} values for each locus (Table 3.6), that resulted 0.214. This value fell down in this specific range 0.15 to 0.25 as exactly described by Wright (1978) qualitative guidelines. The overall F_{ST} significant value indicates a substantial genetic structuring of *Myzus persicae* samples.

Results from pairwise F_{ST} values between populations are shown in Table 3.7. They were all significant except for the following comparisons: San Prospero-109/CesenaB-92, San Martino in Fiume-113/San Prospero-109; San Prospero-109/Bologna-142; San Martino in fiume-113/CesenaB-92, San Prospero-109/Martorano-112 and San Martino in fiume-113/Bologna-142, which had a low F_{ST} value range among 0,109 to 0,040 (Table 3.7). The San Salvo-114 and the Bagnacavallo-133 population showed most differentiation in pairwise comparisons with the other populations, with the highest pairwise F_{ST} value resulting from the comparison between these two populations ($F_{ST} = 0.334$).

The populations which do not appear structured (NS) are those that show the greatest number of migrants in contrast with the structured populations. Absence of migration is another assumption of HWE, as it is able to alter it, being responsible for a marked change in allele frequencies. For this reason, if migration is present, there will be gene flow between populations, bringing alleles or genes from one population to another. Gene flow can be quantified indirectly using F_{ST} estimates. All field populations with NS show a general high number of migrants N_m ranging from 7.20 to 4.00 (Table 3.8).

On the contrary, the N_m rates are very low between the structured populations, wherein the values are near 0.

Pairwise Population Matrix of F_{ST} Values for Total

Populations	Cerignola-145	San Prospero-109	San Martino in Fiume-113	Roma-155	CesenaB-92	CesenaA-125	Imola-127	Martorano-112	Bagnocavallo-133	Pievesestina-102	Bologna-142	San Salvo-114
Cerignola-145		**	**	***	**	***	***	**	***	***	**	***
San Prospero-109	0,095		NS	**	NS	*	*	NS	***	***	NS	***
San Martino in Fiume-113	0,058	0,057		***	NS	**	**	*	***	***	NS	***
Roma-155	0,106	0,092	0,097		*	***	***	***	***	***	***	***
CesenaB-92	0,118	0,109	0,067	0,117		**	**	*	***	**	*	**
CesenaA-125	0,079	0,075	0,065	0,122	0,107		***	*	***	**	**	***
Imola-127	0,109	0,081	0,075	0,123	0,140	0,109		***	***	***	***	***
Martorano-112	0,056	0,046	0,043	0,101	0,086	0,047	0,098		***	***	*	***
Bagnocavallo-133	0,226	0,188	0,195	0,268	0,257	0,236	0,204	0,189		***	***	***
Pievesestina-102	0,179	0,153	0,131	0,220	0,192	0,216	0,211	0,154	0,330		***	***
Bologna-142	0,055	0,057	0,040	0,113	0,094	0,057	0,095	0,052	0,195	0,145		***
San Salvo-114	0,163	0,153	0,154	0,154	0,216	0,160	0,173	0,147	0,334	0,330	0,156	

Table 3.7. Pairwise populations matrix of F_{ST} values. F_{ST} values are shown below the diagonal, above the diagonal the significance value of F_{ST} with its probability are shown (P < 0,05 *; P < 0,01 **; P < 0,001 ***). NS: not significant.

Number of Migrants (Nm)											
Populations	San Prospero-109	Martorano-112	San Martino in Fiume-113	San Salvo-114	CesenaA-125	Imola-127	Bagnocavallo-133	Bologna-142	Cerignola-145	Roma-155	CesenaB-92
Pievesestina-102	0,890	0.82	1.04	0.27	0,520	0.54	0.25	0.91	0.69	0,510	0,540
San Prospero- 109		5.75	4.00	0.82	2,360	2.13	0.65	4.05	1.73	1,770	1,980
Martorano-112			5.71	0.82	4,400	1.48	0.61	4.03	3.56	1,430	2,410
San Martino in Fiume-113				0.79	2,720	2.23	0.60	7.20	3.49	1,540	4,960
San Salvo-114					0,730	0.67	0.26	0.78	0.73	0,780	0,480
CesenaA-125						1,280	0.45	3.40	2.07	1,100	1,490
Imola-127							0,560	1,600	1,330	1,110	1,080
Bagnocavallo-133								0,600	0.49	0,380	0.33
Bologna-142									3,870	1,260	2,260
Cerignola-145(Puglia)										1,390	1,510
Roma-155(Lazio)											1,450
CesenaB-92											

Table 3.8. Pairwise estimated number of migrants ($Nm = 1 - F_{ST} / (4 * F_{ST})$).

The assignment test gives information about the genotype similarity between different populations through a logarithmic system. With this analysis it is possible to assign some individuals at populations different from those where they were found. During the assignment test, nine individuals of different populations (San Prospero-109; Martorano-112; San Martino in Fiume-113; San Salvo-114; Bologna-142; Cerignola-145; Roma-155 and CesenaB-92), have been cross-assigned to other samples. Each population described above presents some individuals unrelated with native populations, for example: the specimens San Prospero-109.02 and 109.10 seem to be belong to Martorano-112, while the specimen Martorano-112.02 was assigned at Cerignola-145. Instead the specimens San Martino in Fiume-113.01, 08, 09 and 10 share some features with populations Pievesestina-102; CesenaA-125; Bologna-142 and San Prospero-127, respectively. Not only, there are also other single individuals, among which San Salvo-114.82, CesenaA-125.02, Bologna-142.03 and Bologna-142.05 that were assigned to Roma-155, San Prospero-109, Martorano-112 and San Martino in fiume-113. Some individuals which belong to the same populations, were assigned to others for example: the specimens Cerignola-145.03, 04, 09 and 10 they were assigned a different populations such as San Martino in fiume-113, Bologna-142, Martorano-112 and San Prospero-109; specimens Roma-155.03, 06 and 09 belong to CesenaB-92, Cerignola-145 and San Prospero-109. At last, CesenaB-92 and 92H5 belong to San Martino in Fiume-113 and Bagnacavallo-133. No relationship emerges between populations Pievesestina-102; Bagnacavallo-133 and Imola-127, which did not assign individuals to other populations. Therefore, the 20% of individuals were assigned with more changes to other populations (Table 3.9).

Summary of Population Assignment Outcomes to 'Self' or 'Other' Population (With Leave One Out Option)		
	Pop Self Pop	Other Pop
Pievesestina-102	8	
San Prospero- 109	8	2
Martorano-112	9	1
San Martino in Fiume-113	5	5
San Salvo-114	9	1
CesenaA-125	8	2
Imola-127	10	
Bagnacavallo-133	9	
Bologna-142	8	2
Cerignola-145	6	4
Roma-155	7	3
CesenaB-92	2	2
Total	89	22
Percent	80%	20%

Table 3.9. Summary of population assignment.

The scatter graph (Fig.3.10) shows the distribution of the different field populations and it is possible to observe a non-homogeneous distribution of the collected species.

It must be noted the two blue isolated points on the right in the graphic (Fig.3.10), because are related to the pop.102 (Pievesestina). This peculiar localization could suggest that pop.102 is an isolated population. Therefore this hypothesis is also supported by data shown in Tab. 3.8 in which we have values around zero, indicating the absence of migration from this population to others.

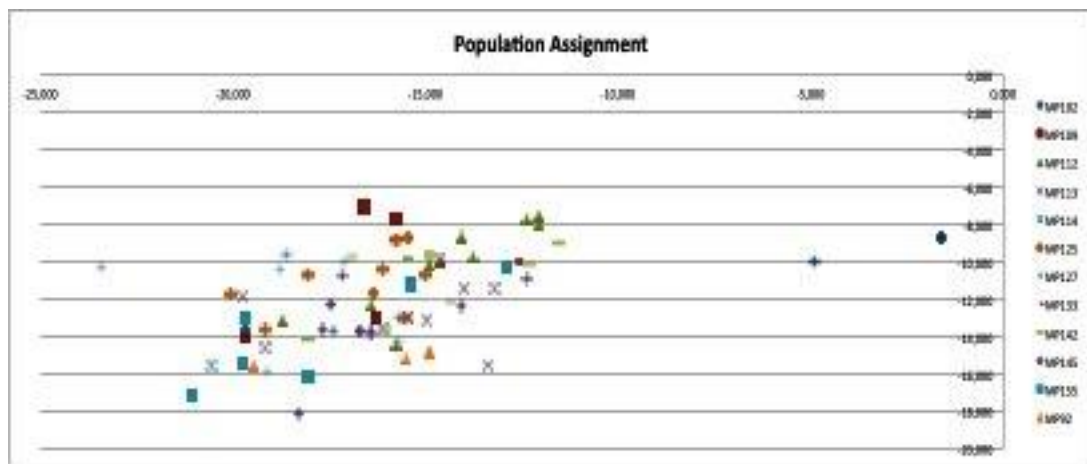


Fig.3.10 Scatter graph of population assignment.

The condition of linkage disequilibrium (LD) occurs when the loci are closely linked, therefore the recombination events will be rare. LD can also be determined by the mixing of populations with different frequencies, by natural selection in favour of some heterozygous genotypes or by random genetic drift. LD value, was calculated in all field populations considering microsatellites and resistance pattern. The values for each population were significant ($P < 0,05$) for M86 locus and R81T ($P = 0,0001$) and M86 and *kdr* ($P = 0,027$). Moreover, the R81T locus was still in linkage with other loci : Myz2 ($P = 0,0003$), Myz25 ($P = 0,0001$) and M49 ($P = 0,001$). Further, the analysis of linkage disequilibrium resulted not significant for four loci M35, M37, M63 and M40 so it means no association between genotype and phenotype resistance *Kdr* (Pyrethroid insecticides) and R81T (neonicotinoid pesticides).

Finally, it was also calculated pairwise F_{ST} values, combining the 8 microsatellite loci with the pattern of resistance loci (*kdr* and R81T) in order to verify if some changes occur on the structuring pattern.

In this case two unstructured population (San Prospero-109 Vs Bologna-142 and San Martino in Fiume-113 Vs Bologna-142) became structured.

Moreover, one structured population (Roma-155 Vs CesenaB-92) has become NS suggesting that resistance could be a parameter which influence the population structure (Table 3.11).

POPULATION	CesenaB-92	San Prospero-109	San Martino in Fiume-113	Roma-155	CesenaA-92	General-125	Imola-127	Montecarlo-112	Bagnacavallo-133	Pievesestina-102	Bologna-142	San Salvo-114
CesenaB-92												
San Prospero-109	0,083											
San Martino in Fiume-113	0,047	0,647										
Roma-155	0,083	0,088	0,087									
CesenaA-92	0,114	0,094	0,064	0,088								
General-125	0,136	0,154	0,118	0,249	0,089							
Imola-127	0,148	0,143	0,119	0,196	0,088	0,112						
Montecarlo-112	0,061	0,058	0,045	0,081	0,091	0,074	0,107					
Bagnacavallo-133	0,126	0,102	0,152	0,241	0,240	0,254	0,207	0,167				
Pievesestina-102	0,188	0,172	0,149	0,281	0,281	0,257	0,214	0,197	0,288			
Bologna-142	0,076	0,071	0,055	0,117	0,080	0,134	0,152	0,082	0,195	0,148		
San Salvo-114	0,188	0,155	0,153	0,157	0,213	0,241	0,235	0,151	0,331	0,328	0,151	

Table 3.11. Pairwise populations matrix of F_{ST} values using microsatellites and resistance pattern (*kdr*/R81T). F_{ST} values are shown below the diagonal, above the diagonal the significance value of F_{ST} with its probability are shown ($P < 0,05$ *; $P < 0,01$ **; $P < 0,001$ ***). NS: not significant.

Furthermore, it has been also carried out an analysis in order to estimate the genetic differences between the populations considering both geographic distance and resistance pattern. The most popular test are Nei and Mantel's test.

Nei's distance (Nei et al. 1983) is a measure that assumes that genetic differences arise due to mutation and genetic drift; genetic differences are usually depicted in a dendrogram as shown in Fig.3.12.

The unrooted Neighbor-Joining dendrogram shows that some genetically related populations are also geographically close, such as Pievesestina-102 and Bagnacavallo-133, and San Prospero-109 and Bologna-142. Other populations with a similar resistance profile (Tab.3.13) are clustered together (e.g. CesenaA-125 and Imola-127, and Roma-155 and San Salvo-114).

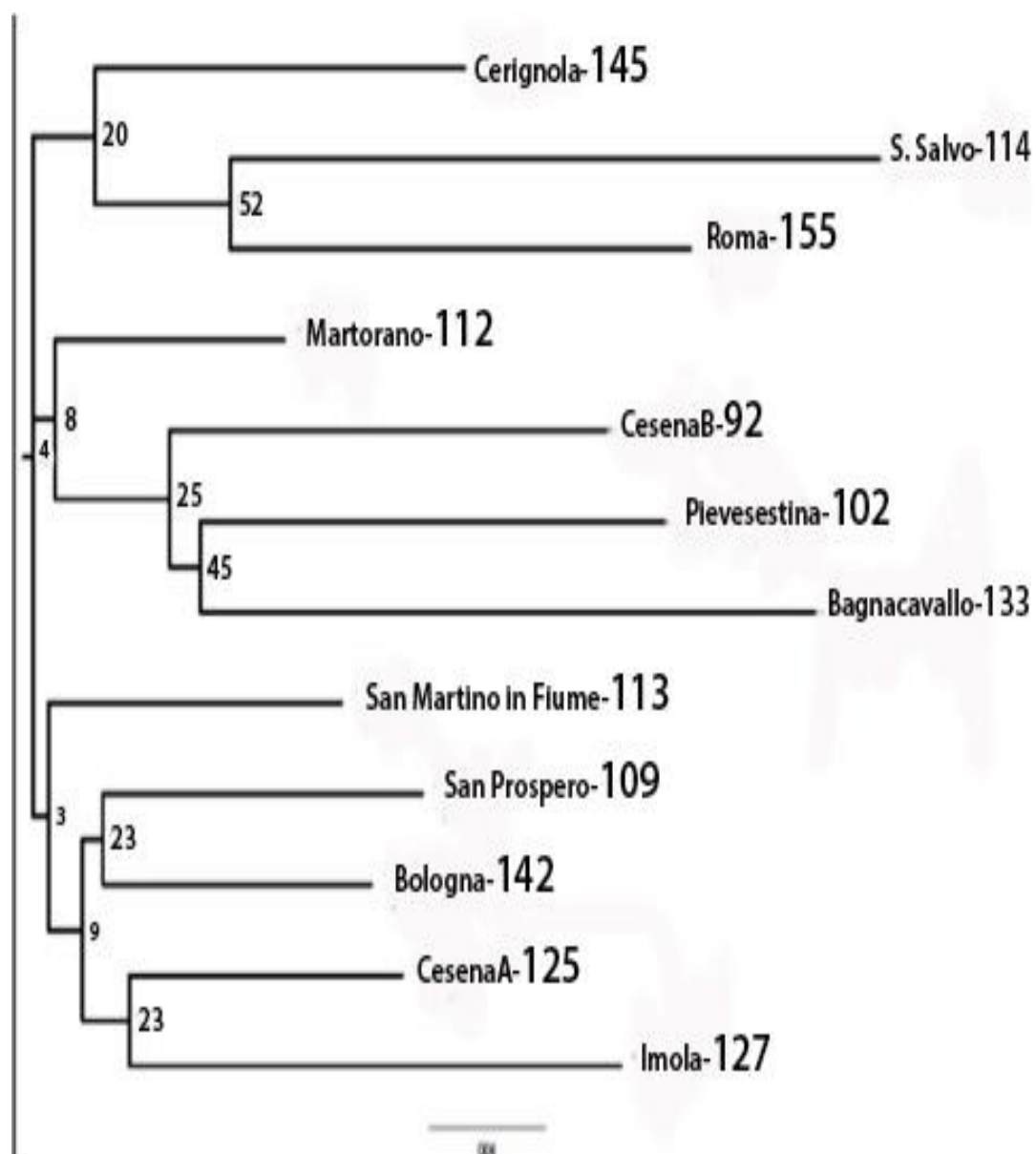


Fig 3.12. Unrooted Neighbor-Joining dendrogram computed on Nei et al. (1983) distances derived among twelve *Myzus persicae* populations; numbers at the nodes indicate bootstrap values

Resistance Loci	Nicotinic Receptor	Sodium channel
Populations	R81T	KDR
CesenaB-92	100% S 0% R	0% S 100% R
Bagnacavallo-133	50% S 50%R	0% S 100% R
Pievesestina-102	50% S 50%R	0% S 100% R
San Martino in Fiume-113	89% S 11% R	28% S 72% R
San Prospero- 109	100% S 0% R	20% S 80% R
Bologna-142	90% S 10% R	0% S 100% R
CesenaA-125	0% S 100% R	25% S 75% R
Imola-127	0% S 100% R	15% S 85% R
Martorano-112	55% S 45% R	10% S 90 % R
San Salvo-114	100% S 0% R	0% S 100% R
Roma-155	65% S 35% R	10% S 90 % R
Cerignola-145	85% S 15% R	20% S 80% R

Table 3.13. Schematic table of allele frequencies related to the resistance loci shared by the populations phylogenetically more closely associated. (S/R) indicate sensibility and resistance phenotype.

The Mantel test (Peakall et.al., 2006) has been used to assess the relationship between geographic patterns and genetic variation in field populations and also to evaluate the existence of a correlation between resistance and microsatellite pattern. Normally the trend should be linear, meaning that all parameters (geographical distance and genetic distance) increase together. The data obtained show a minimal slope with $R^2 = 0,053$; $P < 0,001$, thus evidencing a minimum correlation between geographic distance and genetic variability calculated for pairs of populations ($R_{xy} > 0$; $R_{xy} = 0,231$). This probably suggest that the divergences observed could be due to isolation by distance (Fig.3.14). Obviously we cannot expect high values of correlation because the distances among the collecting sites are not great.

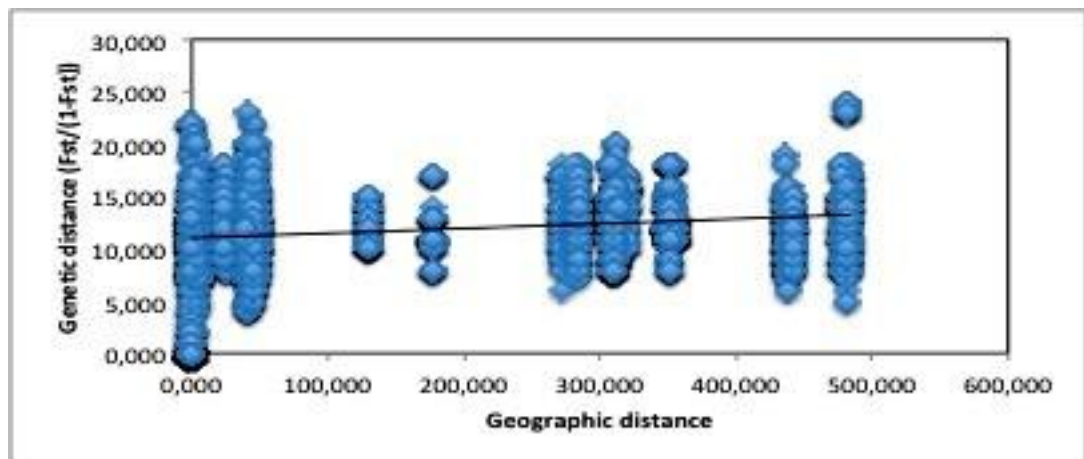


Fig 3.14. The dots correspond to individuals positioned in relation to the geographical distance (X-axis) and genetic (Y-axis). The straight line is an attempt of correlation between these two parameters ($R^2=0,053$; $P<0,001$).

3.1.4 Clonal Structure In Lab-Reared Populations

No intra-population genetic variation was estimated in all analysed populations (see Table 3.17). In order to study genetic and geographic correlation in these population the Mantel test has been performed. This procedure detected a weak but significant positive correlation ($R_{xy}= 0.195$) between geographical (Latitude and Longitude) and genetic distances (F_{ST} of the 47 clonal populations, Fig. 3.15), with a significance level of $P<0.01$.

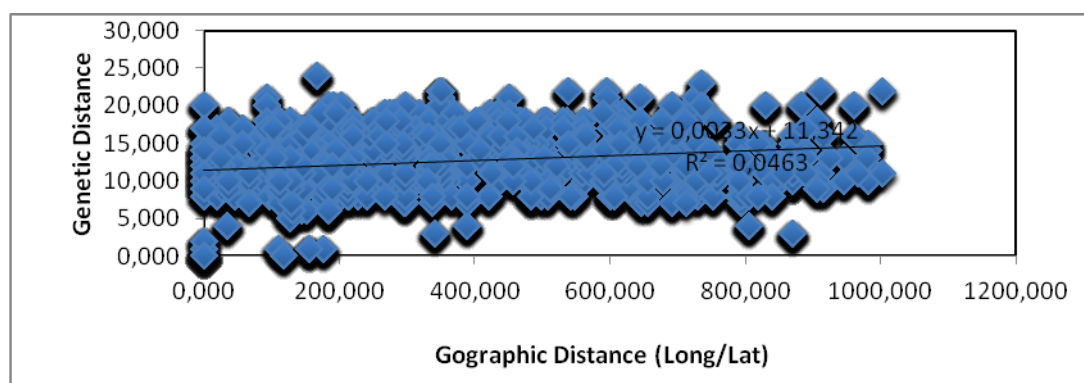


Fig 3.15. Test for isolation by distance using a Mantel Test considering all clonal populations.

This specific test suggest that the observed divergence among populations, could be caused by isolation by distance. The number of alleles for clonal populations ranged from 6 at locus M37 to 22 at locus M49. Total alleles for all loci and all populations were 67, but 23 of them were private alleles, especially found in loci M86, M40, M35, Myz2, Myz25 and M49. Most notably, the population Mp-63 presented only a private allele for locus M49. Therefore, the majority of the samples resulted polymorphic for all loci, except for these clonal lineages: Mp-27H, Mp-70H1, Mp-16, Mp-31, Mp-69, Mp-63, Mp-53, Mp-20, Mp-22, Mp-23, Mp-45, Mp-46, Mp-56, Mp-57, Mp-59, Mp-62, Mp-62H and Mp-83 (Table 3.16).

Percentage of Polymorphic Loci			
Population	%P		
Mp-27H	63,00%	Mp-20	63,00%
Mp-64H	75,00%	Mp-22	63,00%
Mp-70H1	50,00%	Mp-23	63,00%
Mp-48	75,00%	Mp-24	75,00%
Mp-49	75,00%	Mp-34	75,00%
Mp-3	87,50%	Mp-45	63,00%
Mp-37	75,00%	Mp-46	63,00%
Mp-16	63,00%	Mp-51	87,50%
Mp-31	63,00%	Mp-55	75,00%
Mp-69	63,00%	Mp-56	63,00%
Mp-63	63,00%	Mp-57	63,00%
Mp-58	87,50%	Mp-59	63,00%
Mp-76	75,00%	Mp-62	63,00%
Mp-53	37,50%	Mp-62H	63,00%
Mp-33H	75,00%	Mp-68	100,00%
Mp-72	87,50%	Mp-78	87,50%
Mp-26	87,50%	Mp-81	87,50%
Mp-54H2	75,00%	Mp-83	37,50%
Mp-14	100,00%	Mp-84	87,50%
Mp-12	100,00%	Mp-86	100,00%
Mp-17	100,00%	Mp-87	62,50%
Mp-90	62,50%	Mp-93	75,00%
Mp-95	75,00%	Mp-100H	87,50%
Mp-7	75,00%		

Table 3.16. Polymorphic loci considering all clonal lineages.

Allelic richness measured considering all population for each locus had values ranging from 2.000 to 5.52, with Myz2 and Myz25 as the loci with a greater content of alleles. As expected, all clonal species are not in HWE (Table 3.17). In all instance, no null alleles for each single locus was identified. Observed heterozygosity (H_o) over all loci ranged from 1.000 to 0.375, with higher values with respect to expected heterozygosity (H_e), which ranged from 0.188 to 0.438. Probably, the reproduction strategy is the reason of missed linkage between the observed heterozygosity with the expected one.

Table 3.17. Null alleles (Null), Hardy-Weinberg Equilibrium (HWE) observed (Ho)/expected (He) Heterozygosity, allelic richness (Ac),F-statistic between populations (F_{st}) and within populations (F_{is}) per locus and population. Asterisks show data significance (*= $P < 0,05$).

Populations	Statistical Analysis	Loci								Over All Loci for each Pop. Ho/He
		M86	M63	M35	Myz2	Myz25	M40	M49	M37	
Mp-27H	Null HWE Ho/He	No *	No *	No Monomorphic	No *	No Monomorphic	No *	No Monomorphic	No *	0,625/ 0,313
Mp-64H	Null HWE Ho/He	No *	No *	No *	No *	No Monomorphic	No Monomorphic	No *	No *	0,75/0,375
Mp-70H1	Null HWE Ho/He	No *	No *	No Monomorphic	No Monomorphic	No Monomorphic	No *	No *	No *	0,500/0,250
Mp-48	Null HWE Ho/He	No *	No *	No Monomorphic	No *	No Monomorphic	No *	No *	No *	0,75/0,375
Mp-49	Null HWE Ho/He	No *	No *	No *	No *	No Monomorphic	No Monomorphic	No *	No *	0,75/0,375
Mp-3	Null HWE Ho/He	No *	No *	No *	No *	No *	No *	No Monomorphic	No *	0,875/0,438
Mp-37	Null HWE Ho/He	No *	No *	No Monomorphic	No *	No *	No *	No *	No Monomorphic	0,75/0,375
Mp-16	Null HWE Ho/He	No *	No *	No *	No Monomorphic	No *	No *	No Monomorphic	No Monomorphic	0,625/ 0,313
Mp-31	Null HWE Ho/He	No *	No *	No Monomorphic	No Monomorphic	No *	No Monomorphic	No *	No *	0,625/ 0,313
Mp-69	Null HWE Ho/He	No *	No *	No *	No Monomorphic	No Monomorphic	No *	No *	No Monomorphic	0,625/ 0,313
Mp-63	Null HWE Ho/He	No *	No *	No Monomorphic	No *	No Monomorphic	No *	No Monomorphic	No *	0,625/ 0,313
Mp-58	Null HWE Ho/He	No *	No *	No Monomorphic	No *	No *	No *	No *	No *	0,875/0,438
Mp-76	Null HWE Ho/He	No *	No *	No *	No Monomorphic	No *	No *	No *	No Monomorphic	0,75/0,375
Mp-53	Null HWE Ho/He	No *	No Monomorphic	No Monomorphic	No *	No *	No Monomorphic	No Monomorphic	No Monomorphic	0,375/0,188
Mp-33H	Null HWE Ho/He	No *	No *	No Monomorphic	No *	No Monomorphic	No *	No *	No *	0,75/0,375
Mp-72	Null HWE Ho/He	No *	No Monomorphic	No *	No *	No *	No *	No *	No *	0,875/0,438
Mp-26	Null HWE Ho/He	No *	No *	No *	No *	No *	No *	No Monomorphic	No *	0,875/0,438
Mp-54H2	Null HWE Ho/He	No *	No *	No Monomorphic	No *	No Monomorphic	No *	No *	No *	0,75/0,375
Mp-14	Null HWE Ho/He	No *	No *	No *	No *	No *	No *	No *	No *	1,000/0,500
Mp-12	Null HWE Ho/He	No *	No *	No *	No *	No *	No *	No *	No *	1,000/0,500
Mp-17	Null HWE Ho/He	No *	No *	No *	No *	No *	No *	No *	No *	1,000/0,500
Mp-20	Null HWE Ho/He	No *	No *	No Monomorphic	No *	No Monomorphic	No Monomorphic	No *	No *	0,625/ 0,313
Mp-22	Null HWE Ho/He	No Monomorphic	No *	No *	No *	No Monomorphic	No Monomorphic	No *	No *	0,625/ 0,313
Mp-23	Null HWE Ho/He	No *	No *	No Monomorphic	No *	No *	No Monomorphic	No *	No Monomorphic	0,625/ 0,313

Mp-24	Null HWE Ho/He	No *	No *	No *	No *	No Monomorphic	No Monomorphic	No *	No *	0,75/0,375
Mp-34	Null HWE Ho/He	No *	No *	No Monomorphic	No *	No Monomorphic	No *	No *	No *	0,75/0,375
Mp-45	Null HWE Ho/He	No *	No *	No Monomorphic	No Monomorphic	No Monomorphic	No *	No *	No *	0,625/ 0,313
Mp-46	Null HWE Ho/He	No *	No *	No *	No *	No Monomorphic	No Monomorphic	No *	No *	0,625/ 0,313
Mp-51	Null HWE Ho/He	No *	No *	No *	No *	No Monomorphic	No *	No *	No *	0,875/0,438
Mp-55	Null HWE Ho/He	No *	No *	No Monomorphic	No *	No *	No *	No *	No *	0,75/0,375
Mp-56	Null HWE Ho/He	No Monomorphic	No *	No Monomorphic	No *	No *	No *	No *	No Monomorphic	0,625/ 0,313
Mp-57	Null HWE Ho/He	No *	No *	No Monomorphic	No *	No Monomorphic	No Monomorphic	No *	No *	0,625/ 0,313
Mp-59	Null HWE Ho/He	No Monomorphic	No *	No Monomorphic	No *	No *	No *	No *	No Monomorphic	0,625/ 0,313
Mp-62	Null HWE Ho/He	No *	No Monomorphic	No Monomorphic	No *	No Monomorphic	No *	No *	No *	0,625/ 0,313
Mp-62H	Null HWE Ho/He	No *	No Monomorphic	No Monomorphic	No *	No Monomorphic	No *	No *	No *	0,625/ 0,313
Mp-68	Null HWE Ho/He	No *	No *	No *	No *	No *	No *	No *	No *	1,000/0,500
Mp-78	Null HWE Ho/He	No *	No *	No *	No *	No *	No *	No *	No Monomorphic	0,875/0,438
Mp-81	Null HWE Ho/He	No Monomorphic	No *	No *	No *	No *	No *	No *	No *	0,875/0,438
Mp-83	Null HWE Ho/He	No *	No Monomorphic	No Monomorphic	No Monomorphic	No Monomorphic	No *	No *	No Monomorphic	0,375/0,188
Mp-84	Null HWE Ho/He	No *	No *	No *	No *	No Monomorphic	No *	No *	No *	0,875/0,438
Mp-86	Null HWE Ho/He	No	No	No	No	No	No	No	No	1,000/0,500
Mp-87	Null HWE Ho/He	No *	No *	No Monomorphic	No Monomorphic	No *	No *	No *	No *	0,625/ 0,313
Mp-90	Null HWE Ho/He	No *	No *	No Monomorphic	No Monomorphic	No *	No Monomorphic	No *	No *	0,625/ 0,313
Mp-93	Null HWE Ho/He	No *	No *	No *	No *	No Monomorphic	No Monomorphic	No *	No *	0,75/0,375
Mp-95	Null HWE Ho/He	No Monomorphic	No *	No *	No Monomorphic	No *	No *	No *	No *	0,75/0,375
Mp-100H	Null HWE Ho/He	No *	No *	No Monomorphic	No *	No *	No *	No *	No *	0,875/0,438
Mp-7	Null HWE Ho/He	No *	No *	No *	No *	No *	No Monomorphic	No *	No Monomorphic	0,75/0,375
Overall pop for each Locus Allelic Richness	Ac	2.000	2.000	2.000	5.052	4.294	1.000	2.000	1.000	
F- Statistic All Pops	F _{ST}	0,479	0,425	0,625	0,470	0,634	0,527	0,516	0,467	
	F _{IS}	-1,000	-1,000	-1,000	-1,000	-1,000	-1,000	-1,000	-1,000	

The F_{ST} matrix as shown in the table 3.18, shows a strong inter-clonal variability, with most clonal populations presenting a significant population structuring: this suggest a negligible gene flow and a considerable genetic variability between clones. Furthermore, in table 3.18 eight NS values are highlighted, indicating that there are the same clones in different population which are: Mp-64H vs Mp-49, Mp-3 vs Mp-26, Mp-49 vs Mp-51, Mp-51 vs Mp-64H, Mp-54H2 vs Mp-33H, Mp-12 vs Mp-14, Mp-34 vs Mp-33H and Mp-62 vs Mp-62H.

Migration rate estimates (N_m) are very low in all populations as shown in matrix 3.19 even if, it is possible to note a few migrants ranging from 3.00 to 1.00 through populations with same genotypes. Effectively, this situation could indicate the possibility that some individual move between populations, before their collection from the field. Table 3.19 contains some numbers in bold that represent the real migrants' flow, between the different populations for all loci.

Table 3.18. Pairwise populations matrix of F_{ST} values for clonal populations. F_{ST} values are shown below the diagonal, above the diagonal comparisons not significant (NS) are highlighted.

Pairwise Clonal Population Fst Values																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																								
	Mp-27H	Mp-64H	Mp-70H1	Mp-48	Mp-49	Mp-3	Mp-37	Mp-16	Mp-31	Mp-69	Mp-63	Mp-58	Mp-76	Mp-53	Mp-33H	Mp-72	Mp-26	Mp-54H2	Mp-14	Mp-12	Mp-17	Mp-20	Mp-22	Mp-23	Mp-24	Mp-34	Mp-45	Mp-46	Mp-51	Mp-55	Mp-56	Mp-57	Mp-59	Mp-62	Mp-62H	Mp-68	Mp-78	Mp-81	Mp-83	Mp-84	Mp-86	Mp-87	Mp-90	Mp-93	Mp-95	Mp-100H	Mp-7																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																									
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Mp-64H	0,417				NS																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																			
Mp-70H1	0,448	0,450																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																						
Mp-48	0,467	0,248	0,533																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																					
Mp-49	0,450	0,033	0,483	0,248																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																				
Mp-3	0,383	0,400	0,317	0,417	0,400																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																			

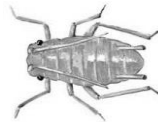
Table 3.19. Pairwise estimated number of migrants ($Nm=1-F_{ST})/(4*F_{ST})$. among the different clonal populations for all loci. Here, are listed all 46 populations.

Clonal Pop.	Mp-64H	Mp-70H1	Mp-48	Mp-49	Mp-3	Mp-37	Mp-16	Mp-31	Mp-69	Mp-63	Mp-58	Mp-76	Mp-53	Mp-33H	Mp-72	Mp-26	Mp-54H2	Mp-14	Mp-12	Mp-17	Mp-20	Mp-22	Mp-23	Mp-24	Mp-34	Mp-45	Mp-46	Mp-51	Mp-55	Mp-56	Mp-57	Mp-59	Mp-62	Mp-62H	Mp-68	Mp-78	Mp-81	Mp-83	Mp-84	Mp-86	Mp-87	Mp-90	Mp-93	Mp-95	Mp-100H	Mp-7
Mp-64H	0.18	0.16	0.16	0.16	0.21	0.25	0.16	0.19	0.17	0.14	0.30	0.23	0.18	0.23	0.23	0.21	0.21	0.25	0.25	0.41	0.19	0.18	0.18	0.17	0.21	0.15	0.19	0.21	0.31	0.25	0.15	0.25	0.15	0.25	0.30	0.33	0.14	0.23	0.27	0.23	0.21	0.20	0.31	0.38	0.18	
Mp-70H1		0.16	0.38	3.00	0.19	0.20	0.15	0.20	0.16	0.23	0.30	0.18	0.11	0.20	0.18	0.19	0.20	0.27	0.25	0.29	0.14	0.18	0.17	0.19	0.20	0.15	0.15	3.25	0.21	0.25	0.28	0.25	0.17	0.17	0.22	0.25	0.25	0.12	0.22	0.35	0.23	0.15	0.25	0.19	0.25	0.19
Mp-48			0.13	0.14	0.25	0.36	0.23	0.32	0.15	0.11	0.34	0.25	0.11	0.28	0.21	0.25	0.28	0.21	0.19	0.25	0.13	0.16	0.23	0.28	0.25	0.16	0.25	0.20	0.23	0.25	0.10	0.25	0.16	0.15	0.25	0.23	0.21	0.09	0.25	0.30	0.13	0.13	0.17	0.16	0.25	0.18
Mp-49				0.38	0.18	0.18	0.14	0.14	0.16	0.25	0.20	0.18	0.11	0.17	0.18	0.18	0.17	0.22	0.22	0.27	0.14	0.17	0.14	0.17	0.17	0.14	0.14	0.41	0.18	0.18	0.21	0.18	0.17	0.17	0.22	0.25	0.23	0.12	0.20	0.27	0.17	0.15	0.19	0.18	0.19	0.18
Mp-3					0.19	0.18	0.14	0.17	0.15	0.23	0.25	0.17	0.10	0.18	0.18	0.19	0.18	0.25	0.23	0.27	0.14	0.17	0.15	0.18	0.18	0.15	0.14	1.63	0.19	0.21	0.28	0.21	0.17	0.17	0.22	0.23	0.23	0.12	0.20	0.32	0.20	0.15	0.23	0.19	0.22	0.18
Mp-37						0.30	0.50	0.30	0.20	0.21	0.39	0.30	0.16	0.30	0.35	3.50	0.27	0.38	0.38	0.42	0.33	0.27	0.21	0.33	0.25	0.33	0.30	0.23	0.30	0.23	0.18	0.23	0.30	0.25	0.94	0.27	0.35	0.16	0.27	0.42	0.25	0.27	0.23	0.36	0.27	0.23
Mp-16							0.28	0.31	0.31	0.16	0.41	0.38	0.17	0.38	0.23	0.30	0.33	0.29	0.25	0.32	0.21	0.21	0.34	0.30	0.33	0.20	0.31	0.25	0.33	0.31	0.14	0.31	0.18	0.17	0.29	0.36	0.27	0.16	0.36	0.39	0.23	0.20	0.23	0.23	0.33	0.19
Mp-31								0.31	0.17	0.15	0.30	0.20	0.13	0.20	0.30	0.50	0.20	0.36	0.27	0.30	0.16	0.16	0.17	0.31	0.17	0.17	0.21	0.19	0.20	0.17	0.14	0.17	0.18	0.16	0.33	0.17	0.27	0.10	0.21	0.30	0.14	0.15	0.17	0.21	0.23	0.23
Mp-69									0.19	0.14	0.75	0.23	0.11	0.31	0.20	0.30	0.31	0.27	0.23	0.30	0.17	0.19	0.19	0.23	0.28	0.15	0.28	0.27	0.39	0.23	0.13	0.23	0.18	0.17	0.30	0.21	0.27	0.10	0.20	0.33	0.21	0.18	0.20	0.21	0.30	0.18
Mp-63										0.15	0.25	0.39	0.10	0.20	0.19	0.20	0.17	0.25	0.23	0.27	0.17	0.17	0.25	0.18	0.20	0.18	0.21	0.20	0.21	0.19	0.12	0.19	0.14	0.14	0.25	0.25	0.25	0.11	0.21	0.27	0.18	0.19	0.20	0.23	0.23	0.14
Mp-58											0.21	0.20	0.08	0.15	0.20	0.21	0.14	0.27	0.27	0.33	0.18	0.17	0.14	0.17	0.15	0.21	0.17	0.25	0.16	0.17	0.19	0.17	0.25	0.25	0.22	0.21	0.30	0.12	0.21	0.36	0.18	0.19	0.18	0.23	0.19	0.14
Mp-76												0.33	0.15	0.46	0.27	0.39	0.41	0.38	0.34	0.47	0.21	0.25	0.30	0.30	0.41	0.25	0.38	0.35	0.46	0.38	0.20	0.38	0.30	0.27	0.38	0.29	0.44	0.14	0.32	0.54	0.38	0.25	0.33	0.36	0.50	0.23
Mp-53													0.15	0.38	0.30	0.30	0.30	0.29	0.32	0.44	0.25	0.25	0.28	0.30	0.33	0.34	0.34	0.22	0.30	0.34	0.14	0.34	0.25	0.25	0.35	0.41	0.33	0.19	0.46	0.44	0.23	0.34	0.23	0.30	0.36	0.19
Mp-33H														0.16	0.18	0.16	0.17	0.18	0.18	0.20	0.10	0.09	0.13	0.20	0.16	0.10	0.10	0.13	0.13	0.13	0.09	0.13	0.09	0.09	0.18	0.16	0.17	0.08	0.21	0.20	0.12	0.09	0.11	0.13	0.17	0.15
Mp-72															0.22	0.30	3.00	0.27	0.29	0.35	0.18	0.23	0.25	0.25	1.00	0.21	0.31	0.25	0.33	0.31	0.14	0.31	0.23	0.21	0.29	0.33	0.27	0.13	0.36	0.35	0.31	0.21	0.21	0.23	0.36	0.20
Mp-26																0.39	0.20	0.47	0.42	0.38	0.21	0.18	0.20	0.33	0.20	0.27	0.21	0.21	0.23	0.23	0.21	0.23	0.27	0.27	0.38	0.22	0.32	0.13	0.29	0.38	0.17	0.25	0.27	0.30	0.29	0.36
Mp-54H2																	0.27	0.38	0.38	0.42	0.33	0.27	0.21	0.33	0.25	0.33	0.30	0.23	0.30	0.23	0.20	0.23	0.30	0.25	0.75	0.27	0.35	0.16	0.27	0.42	0.25	0.27	0.23	0.36	0.27	0.23
Mp-14																		0.25	0.27	0.32	0.17	0.21	0.23	0.25	0.75	0.18	0.28	0.25	0.30	0.28	0.14	0.28	0.21	0.20	0.27	0.30	0.25	0.13	0.36	0.32	0.25	0.18	0.20	0.20	0.33	0.20
Mp-12																			1.33	0.44	0.23	0.25	0.33	0.39	0.27	0.30	0.23	0.31	0.35	0.33	0.27	0.33	0.25	0.27	0.44	0.27	0.47	0.14	0.31	0.50	0.30	0.30	0.35	0.39	0.42	0.29
Mp-17																				0.50	0.23	0.30	0.30	0.35	0.29	0.30	0.23	0.29	0.35	0.36	0.25	0.36	0.25	0.27	0.44	0.29	0.47	0.15	0.31	0.44	0.33	0.30	0.32	0.39	0.42	0.27
Mp-20																					0.30	0.41	0.27	0.35	0.35	0.41	0.54	0.34	0.35	0.46	0.27	0.46	0.33	0.33	0.44	0.42	1.88	0.23	0.38	0.57	0.33	0.30	0.39	0.58	0.54	0.29
Mp-22																						0.21	0.15	0.16	0.18	0.23	0.21	0.19	0.28	0.16	0.13	0.16	0.19	0.19	0.33	0.30	0.23	0.18	0.19	0.27	0.21	0.36	0.18	0.25	0.20	0.14
Mp-23																							0.17	0.16	0.23	0.17	0.23	0.25	0.31	0.28	0.16	0.28	0.15	0.15	0.30	0.38	0.30	0.13	0.20	0.25	0.23	0.19	0.20	0.20	0.27	0.14
Mp-24																								0.31	0.13	0.31	0.15	0.16	0.27	0.25	0.27	0.10	0.30	0.36	0.19	0.16	0.20	0.23	0.19	0.16	0.20	0.23	0.30	0.16		
Mp-34																									0.27	0.28	0.25	0.22	0.23	0.28	0.15	0.28	0.23	0.25	0.39	0.22	0.36	0.13	0.41	0.50	0.17	0.17	0.20	0.27	0.30	0.23
Mp-45																										0.21	0.28	0.25	0.38	0.34	0.15	0.34	0.20	0.21	0.32	0.30	0.30	0.14	0.36	0.35	0.34	0.21	0.23	0.25	0.41	0.19
Mp-46																											0.25	0.17	0.17	0.23	0.13	0.23	0.31	0.31	0.33	0.23	0.33	0.13	0.33	0.54	0.17	0.21	0.20	0.34	0.23	0.16
Mp-51																												0.20	0.25	0.21	0.12	0.21	0.25	0.23	0.27	0.27	0.50	0.14	0.27	0.33	0.17	0.23	0.18	0.31	0.38	0.17
Mp-55																													0.30	0.27	0.27	0.27	0.19	0.19	0.27	0.32	0.29	0.14	0.23	0.38	0.27	0.20	0.25	0.22	0.29	0.22
Mp-56																														0.34	0.16	0.34	0.17	0.18	0.39	0.33	0.30	0.14	0.23	0.32	0.39	0.34	0.33	0.27	0.46	0.21
Mp-57																															0.17	0.17	0.18	0.19	0.30	0.30	0.33	0.12	0.30	0.41	0.25	0.17	0.31	0.25	0.38	0.17
Mp-59																																0.17	0.16	0.17	0.22	0.17	0.23	0.09	0.18	0.25	0.18	0.14	0.23	0.18	0.21	0.15
Mp-62																																	0.18	0.19	0.30	0.30	0.33	0.12	0.30	0.41	0.25	0.17	0.31	0.25	0.38	0.17
Mp-62H																																		2.50	0.25	0.18	0.27	0.12	0.27	0.36	0.15	0.21	0.20	0.25	0.33	0.17
Mp-68																																														

3.2 ANALYSIS OF GENOTOXICITY AFTER INSECTICIDE TREATMENTS

In order to assess possible genotoxic effects of pyrethroid treatments, microsatellite variability has also been analysed in specimens treated with *Brigade Flo* the most commercialised insecticide in agricultural field. Pyrethroids are manufactured synthetic neurotoxins which affect both the peripheral and central nervous system of insects, by acting on the voltage-gated sodium channel proteins found in nerve cell membranes (Garcia, 2011). By prolonging the opening of these channels, pyrethroids stimulate nerve cells to produce repetitive discharges, causing paralysis (known as insect 'knockdown') and possible insect death (Gunasekara 2004; Davies et.al., 2007).

No change in the allelic profile has been observed in all animals under the first, second and third toxic effect. Indeed, all individuals exposed to insecticide showed, for all microsatellites used in this study, the same alleles of wild type specimens.



4 DISCUSSION

This study analyzed the genetic structure of lab-reared and field populations of the peach potato aphid *Myzus persicae* mainly collected from its primary host, the peach tree. The goal was to characterize the genetic variability of this aphid species using microsatellites as markers, and to evaluate the impact of the evolutionary forces that potentially affect this variation: drift, migration and selection. The population-scale analysis of microsatellite repeats were identified using the Applied BioSystem Sequencing. This study also showed data attesting a possible linkage between some microsatellite genotypes and two gene involved in pesticide resistance (*kdr* and nicotine receptor R81T) so that microsatellite analysis could become an alternative method to trace insecticide resistance.

Microsatellites are universally known as the most powerful tools for the analysis of genetic biodiversity. Since molecular markers show Mendelian inheritance, it is possible to trace the fingerprint of each organism and determine the evolutionary history of a species by phylogenetic analysis, studies of genetic relationship, population genetic structures and genetic mapping (Hoshino et.al., 2010). Genetic diversity refers to any variation in nucleotides, genes, chromosomes or whole genomes of organisms (Wang et al., 2009a). It can be assessed among different accessions/individuals within the same species (intraspecific), among species (interspecific) and between genus and families (Mittal & Dubey, 2009). Until now, microsatellites are the most successful system used to determine the population structure within and among populations (Wang et al., 2009a) and for revealing inter or intraspecific phylogenetic relationships, even in closely related species (Wang et al., 2009b). Evaluations of population differentiation permit the estimation of the migration rate between populations, assuming that these populations are in equilibrium (e.g., no selection, identical mutation rates and generation time) (Weising et al., 2005). Instead, the phylogenetic relationships reflect the relatedness of a group of species based on a calculated genetic distance in their evolutionary history.

To date, the genome sequencing technologies promise to revolutionize our understanding of genetics, evolution by making feasible to survey a broad spectrum of sequence variation on a population scale.

Concordant with previous reports, the microsatellites have emerged as important sources of functional variation since they are often located both within and around genes, and frequent mutations in their lengths exert quantitative effects on gene function and phenotype, rapidly degrading linkage disequilibrium between markers and traits.

Nevertheless, results illustrated in this PhD thesis can also support the hypothesis that a change in the genetic structure of the peach aphid during the asexual phase

could be biologically explained, through dispersal behavior of the aphids as often discussed in Guillemaud et.al., (2011) and Wilson et.al., (2002).

4.1 GEOGRAPHIC STRUCTURE IN FIELD POPULATIONS OF *Myzus persicae*

Myzus persicae populations collected mainly in Emilia Romagna but also in other Italian regions, displayed an interesting genetic variation. In particular, a great degree of genotypic diversity was observed in all individuals sampled directly from the field. *M. persicae* is thought to be a cyclic parthenogenetic species, with a single sexual generation and many asexual generations. It is unknown whether this species displays polymorphism in its mode of reproduction, with the coexistence of obligate parthenogenetic and holocyclic individuals, as in many other aphid species (Simon JC et al., 2002). It is already known that the mode of reproduction has consequences for the genetic variation of populations (Hartal et al., 1997). In the case of holocycly, two antagonistic effects occur. Asexual generations (reproducing by mitotic parthenogenesis in this taxon) are expected to generate individuals with an identical genetic background, with mutations as the only source of variation. The occurrence of such asexual generations also leads to systematic linkage disequilibrium (LD) and departure from Hardy-Weinberg equilibrium (HWE). By contrast, sexual (panmictic) generation disrupts inter-locus associations, resulting in each individual being genetically different from all others (Guillemaud et.al., 2011). It also re-establishes HWE within a single generation. In the present study, populations of *M. persicae* collected from its primary host generally showed no significant excess of heterozygotes considering all loci for each population. These data suggest an evidence of sexual reproduction, supporting the hypothesis that the populations collected from peach trees in the spring are probably holocyclic. Therefore, the heterozygosity do not influence the genetic variability. However, the microsatellite analysis put in evidence some populations that showed deviation from HWE and also a high substantial number of observed heterozygous genotypes thus suggesting that these populations retain their genotype for a particular locus. This is probably an evidence for events of obligate parthenogenesis in some populations of *M. persicae*, at least on peach trees in spring. Hence, it remains possible that anholocyclic lineages exist during these periods of the year on secondary hosts, as reported for many aphid species displaying host alternation (Simon et al., 2002). Also Guillemaud et.al. (2011) suggested that populations sampled in spring lacking recombination could be produced by anholocyclic lineages that could be at the basis of the

deviation from HWE as observed in the Italian samples. The high level of genetic variability found in *M. persicae* sampled on the fields, are similar to that found in other cyclic parthenogenetic aphids, such as *D. plantaginea* sampled on its primary host (Guillemaud et.al.,2011), *S. avenae* (Jensen Abet.al.,2008) and *R. padi* (Delmotte F et.al.,2002), and also in other cyclic parthenogenetic animals, such as rotifers (e.g. *Brachionus plicatilis* (Müller), (Gomez A et.al.,2000). The latter display high levels of genotypic diversity despite going through numerous parthenogenetic generations each year. It is possible that a single yearly sexual reproduction event is sufficient to generate a high level of genetic variability and to cancel out the genetic signs of clonality, (Guillemaud et.al., 2011).

Another possible explanations on previous findings in aphids about deviation from HWE can include not only heterozygosity but also a Wahlund effect, null alleles, and selection (Papura et.al.,2003). The Wahlund effect is the unintentional pooling of differentiated populations into a single sample, resulting in excess homozygosity, however, this event seem not to happen in this specific case. Null alleles were suspected for two loci (Myz2 and M35) but for only 2 populations San Prospero-109 e Imola-127. Therefore, a specific statistical analysis was carried out to take this possibility into account only for these two populations, bringing to insight that the presence of null alleles probably could be the reason of the deviation from HWE. Genetic variability may result, also, from genetic drift or selection (e.g. adaptation to various secondary host plants) exactly as described in Guillemaud et.al. (2011).

Another key finding of this study was the weak but significant relation between genetic variability and geographical distribution. Classically, spatial genetic differentiation results from the balance between migration and genetic drift (Wright 1969) .Intra-and inter-population genetic differentiation level was detected using the Mantel test. Obviously we cannot expect high values of genetic correlation, because most of the samples (excluding Lazio, Abruzzo and Puglia) were located in Emilia-Romagna, not far each others. This dataset suggest that observed divergence could be weakly connected to isolation by distance: levels of diversity cannot entirely be explained by geographical range confirming that geographical range is not a good general predictor of patterns of genetic diversity (Soltis and Soltis, 1991). A reduced long-range dispersal ability can be invoked to explain these result

Microsatellite analysis has been also carried on in order to investigate metapopulation dynamics and population genetic structure and it will allow us to compare dispersal and population differentiation of two aphid populations at a small spatial scale. The idea behind assignment tests was to use individual genotypes to assign individuals to populations or clusters. Paetkau et al. (1995) developed the first assignment test approach for use on bears. The idea was fairly simple. Given a set of populations, and the allele frequencies of those populations, what was the likelihood

of a given individual genotype in the population in which it was sampled versus its likelihood in the other populations in the set? An individual is assigned to the population for which it has the highest likelihood. In the present data set only 20% of individuals were assigned to other populations, while all other 80% belong to self. The low percentage of assignment support the low migration data that was found during the field populations study. Indeed, the migration rate is probably a parameter able to affect HWE because it could induce a deep change of allele frequencies. Literature data point out that long-distance migration is not so common in *M. persicae*, or that it has a low success rate (Loxdale et.al.,1993). Some studies about various regions of mainland Greece, affirm the occurrence of common genotypes and show that some long distance migration does occur, but the genetic stability of populations in the same locality from year to year suggests that most of the population does not move far (Blackman et al., 2007). Hence, it would have expected that more the populations were geographically close, much higher would have been the value of migration. Indeed, the migration rate was effectively low, thus meaning that the populations were under the *founder effect* but it is more properly correct to talk about of *bottleneck effect* which identifies a particular type of *genetic drift*. A population bottleneck is a sharp reduction in size of a population due to environmental stochastic events (such as earthquakes, floods, fires, or droughts) or human activities. Such events are able to reduce the variation in the gene pool of a population drastically, because when the population was close to extinction, the few survived passed on those genes to their offspring, which became mostly dominant over the generations. If there are not enough individuals survived, or the ones that survived are not able to reproduce sufficiently to reform the species population, the population may go extinct (William et.al., 2009). In consequence of such population size reductions and the loss of genetic variation, the ability of a population to adapt to prevalent environment or selection like climatic change or shift in available resources is reduced. However, depending upon the causes of the bottleneck, the survivors may increase individual fitness, hence improving the traits within the gene pool while shrinking it. Genetic drift can change the proportional distribution of an allele by chance even leading to fixation or loss of alleles. Due to the smaller population size after a bottleneck event, the chance of inbreeding and genetic homogeneity increases and unfavoured alleles can accumulate (Hufbauer et.al., 2004). The results observed in Italian population refer to aphids sampled from the field previously treated with insecticides and therefore subject to high selective pressure. So, if resistance genes are present in the treated populations, then such pesticide treatments may generate strong selection pressure, increasing the frequency of resistance genes in the field aphid population during spring (Guillemaud et.al.,2011).

Considering the pattern of genetic variability overall by means of F-statistics, the field populations seem to be influenced by the gene flow from refuge areas that contribute to increment the genetic variability. Therefore, on the basis of microsatellite analysis the field populations do not appear structured and then the gene flow actively contribute like a source of genetic variability. Population structure was assessed by calculating multilocus F_{ST} values (Weir & Cockerham, 1984). Considering the pattern of genetic variability overall by means of F-statistic, the field populations seem to be influenced by gene flow allowed by the presence of refuge areas that contribute to increment the genetic variability. Microsatellite analysis evidenced that the field populations appear no structured and then the gene flow actively contribute like a source of genetic variability. Ultimately, the overall effect of the gene flow is to increase the polymorphism of a population and, at the same time, to reduce the average genetic differences between populations. This exchange of genes becomes a unifying force that tends to prevent that populations differ from the genetic point of view. Some literature data about the population structure in aphids affirm that the aphids are highly mobile and thus the structure of a population at any one time will depend on the number of immigrants (Blackman, 1972). Moreover, it was described that a sufficient gene flow between populations can slow down or prevent the process of geographic differentiation as evidenced in the data here reported, and leave a signature of little population structure over large areas (Maaïke A. de Jong et.al., 2011). This is commonly observed in flying insect species, specifically in those species that migrate or are good dispersers. Examples include Monarch butterflies *D. plexippus* (Brower AVZ et.al., 1991) bumble bees *Bombus terrestris* (Estoup A et.al.,1996), and dragonflies *Anax junius* (Freeland JR et.al., 2003).

The Neighbor-Joining dendrogram shows that whereas, as expected, some populations are related as a consequence of geographic vicinity, others are clustered together on the basis of similarity resistance profile, independently from their geographical origin. This could suggest that the selection pressure exerted by agrochemicals is a possible unifying forces that probably prevails over the diversifying forces, as the geographical distance or genetic drift. To date, selection in aphids is well documented and it appears that host plant (Carletto J et.al., 2009), and pesticide treatment (Carletto J et.al., 2010) are among the most important selective factors to be taken into account, especially when trying to understand the population genetic structure of aphid species acting as crop pests.

Regarding the balance between the assignment and the number of migrants, an interesting relationship with the resistance phenotype has been identified. This linkage, could mean that the populations with *Kdr* or R81T resistance have a similar genetic structure, so the statistical analysis treats them as if there was a migration between these populations, although geographically it might be unthinkable. The

genetics likeness could be due to the action of insecticides, which selected similar individual in different places. These results could support the bottleneck effect discussed above because the data presented in this PhD thesis were related to aphids sampled from the field previously treated with insecticides and therefore subject to high selective pressure. Finally, another interesting finding was the high isolation of population 102 in contrast with the population 113. They both came from the same area (Cesena province).). This situation could be described first of all the different resistance profiles of the specimens analyzed and were probably caused by different treatments to which these animals were subjected. It could also be explained by genetic drift, due to an obstruction for the migration events.

4.2 LINKAGE DISEQUILIBRIUM: RESISTANCE GENES AND MICROSATELLITE FINGERPRINTING

Myzus persicae is an ideal species to establish if a correspondence exists between the microsatellites genotype and the resistance genes (*kdr* and R81T). This aphid species is found on numerous crops and it is subject to various forms of phytosanitary pressure. The loci used in this study are known to represent three autosomal, (i) Myz2^A - M49^A - M37^A (ii) M35^A - M63^A (iii) M40^A and one X-linked linkage groups (Sloane et al, 2001). Some literature data have showed pairs of loci on the same chromosome and others on different chromosome, however in the last case they were found to be in significant linkage disequilibrium over all genotypes analyzed (Wilson et.al., 2002). This linkage disequilibrium was explained as by physical linkage and population contractions. However, when some loci are in different linkage groups yet they show general LD over all genotypes, their association could result from selective epistasis as described in Wilson et.al., (2002). Analysing eight loci in aphid population considered for this thesis, a linkage disequilibrium between insecticide resistance mutation (*Kdr* and R81T) and M86^X - Myz25^X and Myz2^A, M49^A loci was found. This probably suggests that one mutated allele of nicotine receptor (R81T) was co-located near to M86^X and Myz25^X microsatellites on sexual chromosomes whereas another allele is probably co-localized on autosomes near Myz2^A- M49^A loci on an autosome. The same reasons explain also the linkage with *kdr* mutation and the locus M86X. This data set was in contrast with Zamoum et.al., (2005) who did not find strict association between the different kinds of markers. The hypothesis that could explain these different result could be the sexual recombination as hypothesized in Zamoum et.al. (2005).

These microsatellite loci could be used as markers to trace some phenotypic trait associated with resistance pattern or to determine the presence or absence of an allele recognised as causing resistance. Microsatellites advantage lies in the fact that they can detect several mechanisms against different active substances, in the same individual. It should be noted however that, considering the evolving capacities of this pest and changes in the use of insecticide classes, these markers must be continuously renewed (Séverine Fontaine et.al., 2013).

4.3 CLONAL STRUCTURE OF *Myzus persicae* LAB-REARED POPULATIONS

In Italian population reared in lab for many years, all loci showed consistently negative values of F_{IS} , indicating an excess of heterozygosity and deviation from HWE. This is probably due to the long term effect of obligate parthenogenesis (parthenogenesis as the only form of reproduction), which tends to lead to excess heterozygosity due to the accumulation of mutations without recombination, as described in Guillemaud et.al., (2011). After many rounds of parthenogenetic generations, *M. persicae* populations could have accumulated high levels of heterozygosity through allelic divergence at neutral nuclear loci, as previously proposed for other aphids asexual lineages such as *Ropalosiphon padi* (Simon et al, 1999b; Delmotte et.al, 2001, 2002) *Schyzaphis avenae* and other related species (Wilson et.al, 1999, 2003; Papura et.al, 2003). Therefore, as pointed out by Vorburger et.al., 2003a in *M. persicae*, this appears to be a characteristic of predominantly asexual populations due to the increased heterozygosity of obligate parthenogenesis. A similar finding was observed in the bird cherry-oat aphid *R. padi* (Delmotte et. al., 2002). Moreover, in clonal organisms the number of alleles maintained at a single locus is often strongly affected by drift towards fixation of heterozygosity (Balloux et.al. 2003).

Bottlenecks can also induce genetic drift resulting from random loss of individuals. In clonal organisms, such as in soybean aphid (Orantes, 2011), the amplification and dispersion of beneficial traits selected after constant bottlenecks are an advantage in terms of the survival and adaption of the species. An example is the amplification and local emergence of resistance traits to soybean cultivars (Kim et al. 2008, Mian et al. 2008).

No intra-population genetic variation was found although a low migration rates has been observed. Some studies demonstrated that there is a stronger differentiation among populations early in the season, and a change in the genetic structure of the

aphid during the asexual phase that can be biologically explained through migration behaviour of the aphids. In other words there are some periods of increased migration and colonization by winged forms, commonly associated with high population density, which could potentially increase the clonal diversity (e.g. McVean et al., 1999). Probably samples analysed in this thesis were collected from the fields prematurely with respect of migration (occurring among peach fields) and therefore, this event caused the absence of relationship between lab-reared lineages. Otherwise, the migration movements happened before their collecting but were not sufficient to create genetic variability.

The data collected for this thesis showed the absence of intra-population genetic variability in lab-reared populations while a substantial level was present in the field strains. The absence of variability in clonal populations could be also explained by selective pressure established during the rearing. This force may delete the initial variability encouraging the genotype having the best fitness for lab-rearing conditions. Afterwards, there are differences in 'general vigor' among clones that allow certain clones ("superclones") to increase more rapidly than others (Loxdale et al., 2011). Whatever the selective pressures that cause the ecological success of the dominant clones, in nature they appear to be strong yet unconstant, based on the fast and marked shifts in the relative frequencies of these clones (C. Vorburger, 2006).

The clonal populations have a significant population structuring suggesting a negligible gene flow and a considerable genetic variability. During the analysis of the structure of lab-reared lineages I observed that some populations on tobacco crops showed similar genetic features, probably because they derived from the same host plant. On the other hand, *M. persicae* populations from tobacco showed different features not only from the populations collected from herbaceous crops but also from the same host (tobacco). As described in Margaritopoulos et al. (2007), this probably due to the ability of tobacco aphids to colonize other plants within the vast host range of *M. persicae* (Semtner, Tilson & Dara, 1998; Nikolakakis et al., 2003). Indeed, all the common (anholocyclic) genotypes found on tobacco in Greece and southern Italy were also found on other host plants in tobacco-growing regions (Blackman et al., 2007). Contrasting with previous works reporting host specialization in *Sitobion* (De Barro et al, 1995; Sunnucks et al, 1998; Lushai et al, 2002), none of the most common Italian genotypes was strictly associated with any of the five host plants (peach, aubergine, potato, tobacco and tomato), considered in the present study. This latter observation is in agreement with results obtained in France, where two genotypes of *S. avenae* were mainly found on maize, but also occurred on other species of cereals (Haack et al, 2000). Finally, the genetic structure that has been observed in Italian clonal populations may be due by at least two factors:

- (1) limited migration that is not sufficient to homogenise the genetic structures of populations on a national scale in obligately parthenogenetic aphids, and probably
- (2) the effect of strong selection.

4. 4 GENOTOXIC EVALUATION OF PYRETHROID

According to Ellegren et.al. (2004) estimating microsatellite mutation rates in samples that are exposed to different forms of radiation or toxic compounds could, when properly related to data from control groups, help to evaluate risk assessments. It is well known, that environmental pollution by pesticide residues is a major environmental concern due to the extensive use of these substances in agriculture. In the past decade, indeed, pyrethroids have emerged as a major class of highly active insecticides due to their high bio-efficacy and relatively low toxicity in comparison to organochlorine and organophosphate pesticides, which are more acutely toxic to birds and mammals than the pyrethroids. These chemicals have been largely used for the control agricultural pests, with Lepidoptera representing the highest percentage (40%) of pyrethroid targeted insects, followed by sucking insects and Coleoptera (Wirtz et al., 2009). To date, many studies have been conducted to assess the toxic effects of pyrethroid on organisms in the environmental using indices including mortality, immobilization and growth inhibition. Actually most of the studies regarding the effects of insecticides (included pyrethroids) on parasitoids and other natural enemies have relied on the evaluation of acute toxicity by determination of a median lethal dose (LD50) or concentration (LC50) (Desneux et al., 2007). Estimation of LD50 or LC50 is a simple and a fast approach to compare and evaluate the acute toxicity of insecticides to parasitoids. The insecticides may directly interfere with the efficacy of biological control agents by changing biological traits, such as, fecundity, fertility, longevity, developmental rates, emergence rates, or indirectly, by modifying behavioural traits that will affect the interactions between parasitoids and their hosts. In view of paucity of literature data, the present study tried to assess the genotoxic effects *in vivo* of a synthetic pyrethroid by treating *M. persicae* specimens with a sublethal exposure of Bifenthrin Brigade Flo., the most commercialised insecticide used to control a widely range of pests. Previous studies reported data which evidenced bioaccumulation and high oral toxicity to mammals, as well as being an endocrine disrupter and a neurotoxicant (Scollon et al., 2011). This pyrethroid is also toxic to birds, most aquatic organisms, honeybees and earthworms (Mukherjee et.al., 2010). In order to verify the potential mutagenic action of Bifenthrin two important conditions are required: i) a clonal lineage with a

stable karyotype and ii) a brief exposure time. It is known that the genotoxic effects have a low frequency insurgence and hence, it is important to use specimens with normal karyotype in order to confer the changes of the microsatellites pattern only at pyrethroid treatments. Further, it has been decided to apply a low exposure time in order to further develop a routine *in vitro* assay of genotoxicity, comparable with others methods as cell culture or *in vivo* models as described in Campana et.al. (2000) and Bhunya et.al. (1988).

The results obtained for all 8 analyzed microsatellites revealed no alteration of the allelic profile thus meaning that no DNA damage and hence no genotoxic effect occurred. The treated animals showed the same microsatellite pattern of the specimens used as wild type so we conclude that the dose and the exposure time *in vivo* have no effect of genotoxicity. Probably, a higher concentration and a longer exposure period could determine different results, as described in the fish *Bryconamericus iheringii* (Marinowic et al, 2012). Only further experiments with higher doses and time of exposition will give irrefutable data regarding the genotoxic affects of pyrethroids on aphids.

In any case, microsatellites, given their high mutation rate, will offer a realistic means to study how the overall genomic mutation rate is affected by environmental factors.

4.5 SEQUENCING OF M49^A LOCUS: DUPLICATION OR TRIPLOIDY ?

PCR-based molecular genetic markers are often designed to assay for fragment-size variation amongst alleles at microsatellite loci, and rely on unique DNA sequence flanking independent tandem repeats to attain locus specificity (Tautz, 1989; Weber & May, 1989). In contrast, instances may occur when PCR amplification products are weak or contain unintended background fragments derived from more than one locus (Economou et al., 1990). Genotypes are difficult to determine correctly from these problematic loci, and analysis of data often result in deviations from Hardy–Weinberg equilibrium within natural populations and Mendelian expectations within pedigrees (Terro et.al., 2006).

Analogous difficulties were often encountered when microsatellite markers were developed for species of Lepidoptera (Meglécz et al., 2004), and may in part be due by the movement of transposable elements (Coates et.al., 2009, 2010). Transposable elements are interesting because they are capable of ‘jumping’ amongst loci, and can affect microsatellites by inserting in proximity or by carrying hitchhiking

microsatellites within the TE itself (Yang & Barbash, 2008). Although microsatellites can be frequently associated to flanking regions, sometimes they are destroyed by transposition events. On the other hand, sometimes some small transposons are inserted in tandem inside a persistent one and this event could generate a co-amplification of two fragments (one longer than the other one), so that in the electropherogram profile two peaks are shown. In this connection, it has been carried out a direct DNA sequencing for the product of the microsatellite locus M49, in order to clarify the abnormal peaks that appeared on the electropherogram profile. In some populations, but not in others, it has been observed recurring strange dinucleotides alleles differing in size (one larger than the other) and number (three or four). M49 is a perfect microsatellite (Eder Jorge Oliveira et.al., 2006) because its repeat sequence (CA)_n is not interrupted by any base not belonging to the motif and it is in linkage with autosome 2. In the samples analysed the insight about the possibility of transposable events was very rare, since that a few individuals (8 for each species) were used during the analysis and these specific phenomenon happen very seldom. The main variations of microsatellites at intra-clonal lineages are multiple and often in linkage to their characteristics of sequence, length and tendency to do some replication errors.

So, another possible interpretation to explain this abnormal event, was the occurrence of a "slippage" during PCR or, alternatively, the gene could have somehow been duplicated and moved to a new position on the chromosome, even though the latter event is very difficult to demonstrate because of the same problem discussed above, that is the small number of tested samples. In order to verify the possibility of the slippage event, the PCR annealing conditions have been altered, increasing the temperature so that only a really good primer match would bind to the primer binding site. These technical arrangements would exclude that one of the two alleles of similar size could be an artefact. The experiment was repeated three times, but the alleles appeared every time, so this explanation could be rejected.

The presence of more alleles in the microsatellite profile may also lead to hypothesize a possible trisomy. Literature data highlight that triploid specimens are quite common in some insect species, for example in bark beetles (Lanier et.al., 2008). As described in some papers of Blackman (1980, 1984, 1991), this trisomic situation could be due to a non disjunction of the chromosomes on the metaphase plate during meiosis. This event may probably have occurred in *M. persicae*, which can no longer sexually cross with the original parental species due to the extra chromosomes (High Loxdale, personal communication). This intriguing hypothesis must be rejected because cytogenetic analysis on all populations that showed abnormal peaks, presented a diploid chromosome number with a stable karyotype (2n=12).

Finally, those results could also derive from specific duplication or gene amplification events. Duplication events are quite common in aphids as put in evidence by *Acyrtosiphon pisum* genomic project (GeneBank: ABLF00000000.2) whereas gene amplification related with insecticide resistance was also observed. Examples have been found in the esterase gene family (E4/FE4) gene of *M. persicae* (Bizzaro et.al., 2005) and in a microsatellite duplication at locus Me-3 in the aphid *Metopeurum fuscoviride* (Loxdale et.al., 2011), where a strange allele with 60 (TG) repeats appeared to be originated from a triple duplication of the originally sequenced gene, composed from only 20 repeats. In the latter case, after sequencing, the flanking regions appeared to be the same as the native sequences. Considering that even in the case of M^A49 the sequencing of abnormal showed exactly the same flanking regions but different number of the repeated unit, we could hypothesize that these abnormal alleles may be due to duplication (amplification) events, perhaps a result of unequal crossing over.



5 CONCLUSION

This microsatellite analysis on aphids, has been carried out in order to assess the genetic variability in different lineages of *Myzus persicae*, monitoring also possible linkages between insecticide resistance and microsatellites. Data obtained allow us to quantify the relative importance of selection and drift on peach aphid genetic variability. Obviously, the potential “Achilles heel” is and will be, the control strategies against *M. persicae*, because the adaptation of this aphid at any site may lead to the failure of control everywhere else. However, in this thesis one important finding was the identification of a linkage between microsatellite and some resistant loci. It will be interesting in future studies to examine in depth other European populations sampled in nature but which are not subject to treatment. Even if the scored differentiation on the Italian field population are generally low, interesting data on population structuring diversity emerges. Although reductions in genetic diversity are generally considered detrimental, the Argentine ant, *Linepithema humile*, constitutes an example of how a genetic bottleneck can precede widespread ecological success when they invade a new niche (Tsutsui et al, 2000, 2001). However, aphids, by their asexual mode of reproduction (either partial or complete), their high dispersal capacities through direct flights or trade exchanges and the existence of 'superclones' seem to be particularly well suited to rapid conquest of new habitats. Since asexual reproduction occurs most of the year in *M. persicae* probably strong selective forces together with the absence of genetic recombination could have major effects on the population genetic structure: departure from Hardy–Weinberg equilibrium, over- representation of some clones, excess of heterozygosity. Moreover, it has often been assumed that, in clonal lineages, the genetic diversity should be related proportionally to spatial scale and the less similar they should be a phenomenon known as isolation by distance (IBD). However the reality is usually more complex, and depends on flight behaviour of the species in question (Chapman et.al.2002).

Molecular advances, especially relating to direct sequencing of DNA and determination of DNA allele size in population genetic studies, and single nucleotide polymorphisms will quickly and efficiently reveal exciting aspects of the evolutionary relationships of aphids, not only with each other but also pertaining to their host plants (Stoneking et.al., 2001). For example, these approaches could be used to shed light on the reason at the basis of the genetic variability found in Abruzzo-Lazio and Puglia, the populations most far away from the others observed in this thesis. Therefore, using sensitive identification of these variants repeats in large-scale next-generation sequencing efforts will enable more comprehensive association studies capable of revealing previously invisible associations in the future.

Finally, detailed studies should be further conducted in order to go in depth regarding the source(s) of genetic variability in *Myzus persicae* populations and to explain the biological and genetic reasons for the ecological success of introduced genotypes. It will also be intriguing to follow the fate of the clones that currently have wide geographic and ecological ranges into the future, to establish whether there is evidence of an increased role for niche specialization and recombination as evolution proceeds. The distribution of genetic variation in space is a prime factor to consider in the conservation and management of natural populations.

After all, an another interesting prospective, will be to try to sequence all the introns of the populations most far away in this work, in order to identify if exist some independent intronic mutations.

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Supplementary Data

Table A. Alleles size of all field population

Populations	Mp-145	Mp-109	Mp-113	Mp-155	Mp-92	Mp-125	Mp-127	Mp-112	Mp-133	Mp-102	Mp-142	Mp-114				
Loci	M86	M63	M35	Myz2	Myz25	M40	M49	M37								
	Allele1	Allele2	Allele1	Allele2	Allele1	Allele2	Allele1	Allele2	Allele1	Allele2	Allele1	Allele2	Allele1	Allele2		
MP145	95	129	169	179	171	181	200	204	118	140	128	128	160	196	158	160
MP145	105	131	177	181	181	181	200	200	116	116	120	128	156	196	160	160
MP145	107	131	175	177	173	181	200	200	116	116	120	128	158	162	156	160
MP145	95	105	171	171	181	181	200	200	118	140	124	128	132	158	152	160
MP145	133	133	175	179	181	181	198	204	116	118	128	128	158	158	152	160
MP145	133	133	175	179	181	181	198	204	116	118	128	128	158	158	152	160
MP145	105	133	175	179	181	181	198	204	116	140	128	128	158	158	152	160
MP145	105	133	175	179	181	181	198	204	116	140	128	128	158	158	152	160
MP145	105	129	177	177	173	181	200	200	118	120	120	128	158	186	160	160
MP145	95	131	171	175	181	191	176	192	120	140	120	128	142	144	152	160
MP109	107	143	175	187	181	181	176	176	120	140	124	128	130	158	152	160
MP109	107	143	175	187	181	181	176	176	120	140	124	128	130	158	152	160
MP109	129	131	171	177	177	191	176	188	116	116	124	128	132	204	154	160
MP109	107	143	175	187	181	181	176	176	120	140	124	128	130	158	152	160
MP109	107	143	175	187	181	181	176	176	120	140	124	128	130	158	152	160
MP109	129	129	167	171	191	191	200	200	116	120	120	120	144	184	154	154
MP109	129	129	167	171	191	191	200	200	116	120	120	120	144	184	154	154
MP109	129	129	167	171	191	191	200	200	116	120	120	120	144	184	154	154
MP109	129	129	167	171	191	191	200	200	116	120	120	120	144	184	154	154
MP109	129	133	171	175	181	181	176	188	118	118	120	128	130	198	160	160
MP113	93	131	175	179	181	195	182	200	118	120	124	128	148	200	154	160
MP113	131	133	175	177	181	191	200	200	118	140	118	124	142	148	154	160
MP113	129	131	177	187	181	181	200	200	118	118	124	128	162	196	154	160
MP113	117	129	171	191	181	181	176	188	116	120	120	128	144	160	154	160
MP113	129	131	167	177	181	181	200	200	118	118	124	128	140	154	154	160
MP113	131	133	175	177	175	181	176	200	118	140	124	124	140	142	154	160
MP113	131	133	175	177	181	191	200	200	118	140	118	124	142	148	154	160
MP113	107	117	167	181	181	181	188	188	116	116	120	128	146	158	154	160
MP113	101	109	171	175	175	181	188	200	140	140	118	124	158	158	152	160
MP113	105	129	171	189	175	191	176	200	116	118	122	128	158	188	156	160
MP155	105	105	175	177	191	191	190	202	116	120	120	128	142	190	154	160
MP155	105	105	175	177	191	191	190	202	116	120	120	128	142	190	154	160
MP155	129	133	175	177	175	191	188	200	142	142	120	128	144	204	154	154
MP155	105	105	175	177	191	191	190	202	116	120	120	128	142	190	154	160
MP155	107	133	175	177	181	191	200	200	116	120	128	128	160	162	154	160
MP155	107	131	171	175	181	193	198	204	116	116	128	128	142	162	150	160
MP155	105	105	177	179	173	173	190	200	116	120	120	128	144	188	154	160
MP155	105	105	175	177	191	191	190	202	116	120	120	128	142	190	154	160
MP155	107	129	179	189	181	191	200	200	120	122	128	128	132	144	152	160
MP155	105	105	175	177	191	191	190	202	116	120	120	128	142	190	154	160
MP92	131	135	175	177	181	193	188	198	116	118	120	124	142	192	154	154
MP92	131	131	175	177	173	181	188	200	120	142	124	128	160	192	160	160
MP92	131	131	175	177	173	181	188	200	120	142	124	128	160	192	160	160
MP92	129	129	171	177	173	181	192	200	142	142	120	128	136	160	154	154
MP125	117	129	175	179	181	191	188	188	116	140	124	128	156	158	160	160
MP125	111	129	169	171	177	191	198	200	116	142	120	124	158	180	152	154
MP125	117	129	177	177	181	191	188	188	116	140	120	128	156	158	160	160
MP125	117	129	175	179	181	191	188	188	116	140	124	128	158	158	160	160
MP125	117	129	171	177	181	191	188	188	116	140	120	128	156	158	160	160
MP125	129	143	175	177	181	181	176	188	118	140	120	128	156	158	154	160
MP125	129	143	175	187	181	181	176	200	116	140	120	128	156	158	156	160
MP125	117	129	175	179	181	191	188	188	116	140	124	128	142	160	160	160
MP125	129	143	175	177	181	181	176	188	118	140	120	128	160	160	154	160
MP127	105	129	179	187	181	191	176	176	116	116	118	120	144	144	154	160
MP127	105	129	175	189	181	191	176	176	116	118	128	128	144	144	154	154
MP127	133	143	175	187	177	181	188	200	116	118	124	128	144	156	152	160
MP127	93	129	177	187	177	181	200	200	116	116	124	130	144	156	156	160
MP127	105	129	175	189	181	191	176	176	116	118	128	128	144	144	154	154
MP127	93	129	171	179	177	181	200	200	116	116	124	130	144	156	156	160
MP127	93	129	177	187	177	181	200	200	116	116	124	130	144	156	156	160
MP127	133	143	175	187	177	181	188	200	116	118	124	128	144	156	154	160
MP127	93	129	175	187	177	181	200	200	116	116	124	130	144	156	156	160
MP127	97	117	171	179	177	181	200	200	116	116	124	130	144	156	156	160

MP112	129	133	171	175	181	181	176	200	116	140	120	128	134	158	154	160
MP112	95	129	177	189	173	191	200	204	118	120	124	128	156	158	160	160
MP112	129	133	171	175	181	181	176	200	118	140	120	128	134	158	154	160
MP112	131	131	177	191	173	191	176	188	118	120	128	128	158	158	160	160
MP112	129	131	171	175	181	181	176	200	116	116	120	128	134	158	154	160
MP112	129	131	171	177	181	181	176	200	140	140	120	128	134	158	154	160
MP112	129	131	171	177	181	181	176	200	138	140	120	128	134	158	154	160
MP112	117	129	171	191	181	191	176	188	116	120	128	128	144	160	154	160
MP112	117	129	171	191	181	191	176	188	116	120	124	128	144	160	154	160
MP112	95	129	177	189	173	191	176	188	118	120	124	128	158	158	160	160
MP133	129	129	171	195	181	181	200	200	116	116	120	128	134	136	154	160
MP133	129	129	171	195	181	181	200	200	116	116	120	120	134	136	154	160
MP133	129	129	171	195	181	181	200	200	116	116	120	120	134	136	154	160
MP133	129	129	171	195	181	181	200	200	116	116	120	128	134	136	154	160
MP133	129	129	171	195	181	181	200	200	116	116	120	128	134	136	154	160
MP133	129	129	171	195	181	181	200	200	116	116	120	128	134	136	154	160
MP133	129	129	171	195	181	181	200	200	116	116	120	128	134	136	154	160
MP133	129	129	171	195	181	181	200	200	116	116	120	128	134	136	154	160
MP133	129	129	171	195	181	181	200	200	116	116	120	128	134	136	154	160
MP133	129	129	171	195	181	181	200	200	116	116	120	128	134	136	154	160
MP133	129	129	171	195	181	181	200	200	116	116	120	128	134	136	154	160
MP102	93	129	171	175	181	181	200	200	120	120	124	128	142	158	154	160
MP102	93	129	171	175	181	181	200	200	120	120	124	128	142	158	154	160
MP102	93	129	171	175	181	181	200	200	120	120	124	128	142	158	154	160
MP102	93	129	171	175	181	181	200	200	120	120	124	128	142	158	154	160
MP102	93	129	171	175	181	181	200	200	120	120	124	128	140	160	154	160
MP102	93	129	171	175	181	181	200	200	120	120	124	128	142	158	154	160
MP102	93	129	171	175	181	181	200	200	120	120	124	128	142	158	154	160
MP102	93	129	171	175	181	181	200	200	120	120	124	128	142	158	154	160
MP102	93	129	171	175	181	181	200	200	120	120	124	128	142	158	154	160
MP142	105	143	171	187	181	181	200	206	138	140	124	124	132	158	160	160
MP142	143	143	171	175	181	181	176	200	120	138	124	128	132	194	160	160
MP142	129	129	171	177	181	191	200	204	120	140	120	128	158	160	154	154
MP142	131	143	171	177	181	191	200	200	118	140	124	128	148	158	152	160
MP142	105	131	171	171	175	181	176	190	116	116	118	128	156	158	154	160
MP142	129	143	175	175	181	181	188	200	116	140	120	128	160	194	152	160
MP142	105	143	171	187	171	181	200	206	138	140	124	124	132	158	160	160
MP142	131	143	171	177	181	191	200	200	118	140	124	128	148	158	152	160
MP142	129	143	175	175	181	181	188	200	116	140	120	128	160	194	152	160
MP142	129	143	175	175	181	181	188	200	116	140	120	120	160	194	152	160
MP114	107	135	175	177	189	191	198	200	120	142	112	120	134	158	152	160
MP114	107	117	171	175	181	191	176	190	116	116	128	132	148	188	160	160
MP114	107	117	171	175	181	191	176	190	116	116	128	132	148	188	160	160
MP114	107	117	171	175	181	191	176	190	116	116	128	132	148	188	160	160
MP114	107	117	171	175	181	191	176	190	116	116	128	132	148	188	160	160
MP114	107	117	171	175	181	191	176	190	116	116	128	132	148	188	160	160
MP114	107	117	171	175	181	191	176	190	116	116	128	132	148	188	160	160
MP114	107	117	171	175	181	191	176	190	116	116	128	132	148	188	160	160
MP114	107	117	171	175	181	191	176	190	116	116	128	132	148	188	160	160
MP114	107	117	171	175	181	191	176	190	116	116	128	132	148	188	160	160
MP114	107	117	171	175	181	191	176	190	116	116	128	132	148	188	160	160

Table B. Alleles size of all Lab-Reared population

Mp-70H1.04	91	107	177	179	181	181	176	176	116	116	120	124	148	158	160	160
Mp-70H1.05	91	107	177	179	181	181	176	176	116	116	120	124	148	158	160	160
Mp-70H1.06	91	107	177	179	181	181	176	176	116	116	120	124	148	158	160	160
Mp-48.01	93	95	169	189	177	177	192	198	118	118	130	132	132	162	152	160
Mp-48.02	93	95	169	189	177	177	192	198	118	118	130	132	132	162	152	160
Mp-48.03	93	95	169	189	177	177	192	198	118	118	130	132	132	162	152	160
Mp-48.04	93	95	169	189	177	177	192	198	118	118	130	132	132	162	152	160
Mp-48.05	93	95	169	189	177	177	192	198	118	118	130	132	132	162	152	160
Mp-48.06	93	95	169	189	177	177	192	198	118	118	130	132	132	162	152	160
Mp-49.01	93	101	161	171	177	183	176	192	118	118	130	130	112	140	152	160
Mp-49.02	93	101	161	171	177	183	176	192	118	118	130	130	112	140	152	160
Mp-49.03	93	101	161	171	177	183	176	192	118	118	130	130	112	140	152	160
Mp-49.04	93	101	161	171	177	183	176	192	118	118	130	130	112	140	152	160
Mp-49.05	93	101	161	171	177	183	176	192	118	118	130	130	112	140	152	160
Mp-49.06	93	101	161	171	177	183	176	192	118	118	130	130	112	140	152	160
Mp-3.01	129	131	177	179	189	191	176	200	116	120	120	124	158	158	152	154
Mp-3.02	129	131	177	179	189	191	176	200	116	120	120	124	158	158	152	154
Mp-3.03	129	131	177	179	189	191	176	200	116	120	120	124	158	158	152	154
Mp-3.04	129	131	177	179	189	191	176	200	116	120	120	124	158	158	152	154
Mp-3.05	129	131	177	179	189	191	176	200	116	120	120	124	158	158	152	154
Mp-3.06	129	131	177	179	189	191	176	200	116	120	120	124	158	158	152	154
Mp-37.01	127	133	175	189	181	181	176	200	116	120	120	124	158	198	160	160
Mp-37.02	127	133	175	189	181	181	176	200	116	120	120	124	158	198	160	160
Mp-37.03	127	133	175	189	181	181	176	200	116	120	120	124	158	198	160	160
Mp-37.04	127	133	175	189	181	181	176	200	116	120	120	124	158	198	160	160
Mp-37.05	127	133	175	189	181	181	176	200	116	120	120	124	158	198	160	160
Mp-37.06	127	133	175	189	181	181	176	200	116	120	120	124	158	198	160	160
Mp-16.01	129	133	177	189	181	191	176	176	116	142	120	124	158	158	154	154
Mp-16.02	129	133	177	189	181	191	176	176	116	142	120	124	158	158	154	154
Mp-16.03	129	133	177	189	181	191	176	176	116	142	120	124	158	158	154	154
Mp-16.04	129	133	177	189	181	191	176	176	116	142	120	124	158	158	154	154
Mp-16.05	129	133	177	189	181	191	176	176	116	142	120	124	158	158	154	154
Mp-16.06	129	133	177	189	181	191	176	176	116	142	120	124	158	158	154	154
Mp-31.01	129	135	171	177	181	181	176	176	116	142	120	120	158	192	152	160
Mp-31.03	129	135	171	177	181	181	176	176	116	142	120	120	158	192	152	160
Mp-31.04	129	135	171	177	181	181	176	176	116	142	120	120	158	192	152	160
Mp-31.05	129	135	171	177	181	181	176	176	116	142	120	120	158	192	152	160
Mp-31.06	129	135	171	177	181	181	176	176	116	142	120	120	158	192	152	160
Mp-69.01	129	133	161	169	173	181	200	200	142	142	120	124	162	198	160	160
Mp-69.02	129	133	161	169	173	181	200	200	142	142	120	124	162	198	160	160
Mp-69.03	129	133	161	169	173	181	200	200	142	142	120	124	162	198	160	160
Mp-69.04	129	133	161	169	173	181	200	200	142	142	120	124	162	198	160	160
Mp-69.05	129	133	161	169	173	181	200	200	142	142	120	124	162	198	160	160
Mp-69.06	129	133	161	169	173	181	200	200	142	142	120	124	162	198	160	160
Mp-63.01	93	129	177	189	191	191	198	200	118	118	126	130	124	124	152	160
Mp-63.02	93	129	177	189	191	191	198	200	118	118	126	130	124	124	152	160
Mp-63.03	93	129	177	189	191	191	198	200	118	118	126	130	124	124	152	160
Mp-63.04	93	129	177	189	191	191	198	200	118	118	126	130	124	124	152	160
Mp-63.05	93	129	177	189	191	191	198	200	118	118	126	130	124	124	152	160
Mp-63.06	93	129	177	189	191	191	198	200	118	118	126	130	124	124	152	160
Mp-58.01	129	135	171	177	181	181	176	200	116	128	120	130	134	158	152	160
Mp-58.02	129	135	171	177	181	181	176	200	116	128	120	130	134	158	152	160
Mp-58.03	129	135	171	177	181	181	176	200	116	128	120	130	134	158	152	160
Mp-58.04	129	135	171	177	181	181	176	200	116	128	120	130	134	158	152	160
Mp-58.05	129	135	171	177	181	181	176	200	116	128	120	130	134	158	152	160
Mp-58.06	129	135	171	177	181	181	176	200	116	128	120	130	134	158	152	160
Mp-76.01	107	131	175	177	181	191	200	200	116	142	120	124	162	164	160	160
Mp-76.02	107	131	175	177	181	191	200	200	116	142	120	124	162	164	160	160
Mp-76.03	107	131	175	177	181	191	200	200	116	142	120	124	162	164	160	160
Mp-76.04	107	131	175	177	181	191	200	200	116	142	120	124	162	164	160	160
Mp-76.05	107	131	175	177	181	191	200	200	116	142	120	124	162	164	160	160
Mp-76.06	107	131	175	177	181	191	200	200	116	142	120	124	162	164	160	160
Mp-53.01	107	109	175	175	181	181	180	202	116	120	124	124	132	132	154	154
Mp-53.02	107	109	175	175	181	181	180	202	116	120	124	124	132	132	154	154
Mp-53.03	107	109	175	175	181	181	180	202	116	120	124	124	132	132	154	154
Mp-53.04	107	109	175	175	181	181	180	202	116	120	124	124	132	132	154	154
Mp-53.05	107	109	175	175	181	181	180	202	116	120	124	124	132	132	154	154
Mp-53.06	107	109	175	175	181	181	180	202	116	120	124	124	132	132	154	154
Mp-33H.01	107	131	171	175	181	181	200	204	116	116	118	120	146	158	152	160
Mp-33H.02	107	131	171	175	181	181	200	204	116	116	118	120	146	158	152	160
Mp-33H.03	107	131	171	175	181	181	200	204	116	116	118	120	146	158	152	160
Mp-33H.04	107	131	171	175	181	181	200	204	116	116	118	120	146	158	152	160
Mp-33H.05	107	131	171	175	181	181	200	204	116	116	118	120	146	158	152	160
Mp-33H.06	107	131	171	175	181	181	200	204	116	116	118	120	146	158	152	160
Mp-72.01	107	133	177	177	191	193	176	200	116	140	122	124	132	194	154	158
Mp-72.02	107	133	177	177	191	193	176	200	116	140	122	124	132	194	154	158
Mp-72.03	107	133	177	177	191	193	176	200	116	140	122	124	132	194	154	158
Mp-72.04	107	133	177	177	191	193	176	200	116	140	122	124	132	194	154	158
Mp-72.05	107	133	177	177	191	193	176	200	116	140	122	124	132	194	154	158
Mp-72.06	107	133	177	177	191	193	176	200	116	140	122	124	132	194	154	158
Mp-26.01	129	131	177	179	191	193	176	200	116	120	120	124	158	158	152	154
Mp-26.02	129	131	177	179	191	193	176	200	116	120	120	124	158	158	152	154
Mp-26.03	129	131	177	179	191	193	176	200	116	120	120	124	158	158	152	154
Mp-26.04	129	131	177	179	191	193	176	200	116	120	120	124	158	158	152	154

Mp-56.02	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
Mp-56.03	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
Mp-56.04	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
Mp-56.05	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
Mp-56.06	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
Mp-57.01	93	133	171	177	193	193	178	190	118	118	130	130	150	160	152	154
Mp-57.02	93	133	171	177	193	193	178	190	118	118	130	130	150	160	152	154
Mp-57.03	93	133	171	177	193	193	178	190	118	118	130	130	150	160	152	154
Mp-57.04	93	133	171	177	193	193	178	190	118	118	130	130	150	160	152	154
Mp-57.05	93	133	171	177	193	193	178	190	118	118	130	130	150	160	152	154
Mp-57.06	93	133	171	177	193	193	178	190	118	118	130	130	150	160	152	154
Mp-59.01	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
Mp-59.02	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
Mp-59.03	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
Mp-59.04	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
Mp-59.05	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
Mp-59.06	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
Mp-62.01	93	135	177	177	191	191	198	200	116	116	130	134	146	158	152	160
Mp-62.02	93	135	177	177	191	191	198	200	116	116	130	134	146	158	152	160
Mp-62.03	93	135	177	177	191	191	198	200	116	116	130	134	146	158	152	160
Mp-62.04	93	135	177	177	191	191	198	200	116	116	130	134	146	158	152	160
Mp-62.05	93	135	177	177	191	191	198	200	116	116	130	134	146	158	152	160
Mp-62.06	93	135	177	177	191	191	198	200	116	116	130	134	146	158	152	160
Mp-62H.01	93	135	177	177	191	191	198	200	116	116	130	134	146	160	152	160
Mp-62H.02	93	135	177	177	191	191	198	200	116	116	130	134	146	160	152	160
Mp-62H.03	93	135	177	177	191	191	198	200	116	116	130	134	146	160	152	160
Mp-62H.04	93	135	177	177	191	191	198	200	116	116	130	134	146	160	152	160
Mp-62H.05	93	135	177	177	191	191	198	200	116	116	130	134	146	160	152	160
Mp-62H.06	93	135	177	177	191	191	198	200	116	116	130	134	146	160	152	160
Mp-68.01	129	131	177	179	187	189	176	200	116	120	120	124	160	162	152	154
Mp-68.02	129	131	177	179	187	189	176	200	116	120	120	124	160	162	152	154
Mp-68.03	129	131	177	179	187	189	176	200	116	120	120	124	160	162	152	154
Mp-68.04	129	131	177	179	187	189	176	200	116	120	120	124	160	162	152	154
Mp-68.05	129	131	177	179	187	189	176	200	116	120	120	124	160	162	152	154
Mp-68.06	129	131	177	179	187	189	176	200	116	120	120	124	160	162	152	154
Mp-78.01	107	131	175	179	177	181	188	200	118	120	120	126	144	200	160	160
Mp-78.02	107	131	175	179	177	181	188	200	118	120	120	126	144	200	160	160
Mp-78.03	107	131	175	179	177	181	188	200	118	120	120	126	144	200	160	160
Mp-78.04	107	131	175	179	177	181	188	200	118	120	120	126	144	200	160	160
Mp-78.05	107	131	175	179	177	181	188	200	118	120	120	126	144	200	160	160
Mp-78.06	107	131	175	179	177	181	188	200	118	120	120	126	144	200	160	160
Mp-81.01	129	129	177	187	181	191	188	200	116	118	120	130	132	160	154	160
Mp-81.02	129	129	177	187	181	191	188	200	116	118	120	130	132	160	154	160
Mp-81.03	129	129	177	187	181	191	188	200	116	118	120	130	132	160	154	160
Mp-81.04	129	129	177	187	181	191	188	200	116	118	120	130	132	160	154	160
Mp-81.05	129	129	177	187	181	191	188	200	116	118	120	130	132	160	154	160
Mp-81.06	129	129	177	187	181	191	188	200	116	118	120	130	132	160	154	160
Mp-83.01	109	131	175	175	191	191	188	188	120	120	120	130	130	162	160	160
Mp-83.02	109	131	175	175	191	191	188	188	120	120	120	130	130	162	160	160
Mp-83.03	109	131	175	175	191	191	188	188	120	120	120	130	130	162	160	160
Mp-83.04	109	131	175	175	191	191	188	188	120	120	120	130	130	162	160	160
Mp-83.05	109	131	175	175	191	191	188	188	120	120	120	130	130	162	160	160
Mp-83.06	109	131	175	175	191	191	188	188	120	120	120	130	130	162	160	160
Mp-84.01	107	117	175	189	181	191	200	202	116	116	124	130	160	164	150	160
Mp-84.02	107	117	175	189	181	191	200	202	116	116	124	130	160	164	150	160
Mp-84.03	107	117	175	189	181	191	200	202	116	116	124	130	160	164	150	160
Mp-84.04	107	117	175	189	181	191	200	202	116	116	124	130	160	164	150	160
Mp-84.05	107	117	175	189	181	191	200	202	116	116	124	130	160	164	150	160
Mp-84.06	107	117	175	189	181	191	200	202	116	116	124	130	160	164	150	160
Mp-86.01	117	129	175	177	181	191	176	200	116	118	124	130	136	144	152	160
Mp-86.02	117	129	175	177	181	191	176	200	116	118	124	130	136	144	152	160
Mp-86.03	117	129	175	177	181	191	176	200	116	118	124	130	136	144	152	160
Mp-86.04	117	129	175	177	181	191	176	200	116	118	124	130	136	144	152	160
Mp-86.05	117	129	175	177	181	191	176	200	116	118	124	130	136	144	152	160
Mp-86.06	117	129	175	177	181	191	176	200	116	118	124	130	136	144	152	160
Mp-87.01	129	131	171	175	181	181	200	200	118	120	120	130	122	160	152	152
Mp-87.02	129	131	171	175	181	181	200	200	118	120	120	130	122	160	152	152
Mp-87.03	129	131	171	175	181	181	200	200	118	120	120	130	122	160	152	152
Mp-87.04	129	131	171	175	181	181	200	200	118	120	120	130	122	160	152	152
Mp-87.05	129	131	171	175	181	181	200	200	118	120	120	130	122	160	152	152
Mp-87.06	129	131	171	175	181	181	200	200	118	120	120	130	122	160	152	152
Mp-90.01	97	107	171	177	191	191	200	200	120	140	120	120	160	162	152	160
Mp-90.02	97	107	171	177	191	191	200	200	120	140	120	120	160	162	152	160
Mp-90.03	97	107	171	177	191	191	200	200	120	140	120	120	160	162	152	160
Mp-90.04	97	107	171	177	191	191	200	200	120	140	120	120	160	162	152	160
Mp-90.05	97	107	171	177	191	191	200	200	120	140	120	120	160	162	152	160
Mp-90.06	97	107	171	177	191	191	200	200	120	140	120	120	160	162	152	160
Mp-93.01	131	133	177	187	173	181	176	200	140	140	130	130	150	160	152	160
Mp-93.02	131	133	177	187	173	181	176	200	140	140	130	130	150	160	152	160
Mp-93.03	131	133	177	187	173	181	176	200	140	140	130	130	150	160	152	160
Mp-93.04	131	133	177	187	173	181	176	200	140	140	130	130	150	160	152	160
Mp-93.05	131	133	177	187	173	181	176	200	140	140	130	130	150	160	152	160
Mp-93.06	131	133	177	187	173	181	176	200	140	140	130	130	150	160	152	160
Mp-95.01	129	129	171	177	175	191	200	200	116	120	120	130	132	160	154	160

MP-51.01	93	101	161	171	177	181	176	192	118	118	120	130	112	140	152	160
MP-51.02	93	101	161	171	177	181	176	192	118	118	120	130	112	140	152	160
MP-51.03	93	101	161	171	177	181	176	192	118	118	120	130	112	140	152	160
MP-51.04	93	101	161	171	177	181	176	192	118	118	120	130	112	140	152	160
MP-51.05	93	101	161	171	177	181	176	192	118	118	120	130	112	140	152	160
MP-51.06	93	101	161	171	177	181	176	192	118	118	120	130	112	140	152	160
MP-55.01	95	131	171	177	181	181	176	200	120	140	120	120	160	182	152	160
MP-55.02	95	131	171	177	181	181	176	200	120	140	120	120	160	182	152	160
MP-55.03	95	131	171	177	181	181	176	200	120	140	120	120	160	182	152	160
MP-55.04	95	131	171	177	181	181	176	200	120	140	120	120	160	182	152	160
MP-55.05	95	131	171	177	181	181	176	200	120	140	120	120	160	182	152	160
MP-55.06	95	131	171	177	181	181	176	200	120	140	120	120	160	182	152	160
MP-56.01	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
MP-56.02	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
MP-56.03	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
MP-56.04	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
MP-56.05	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
MP-56.06	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
MP-57.01	93	133	171	177	193	193	178	190	118	118	130	130	150	160	152	154
MP-57.02	93	133	171	177	193	193	178	190	118	118	130	130	150	160	152	154
MP-57.03	93	133	171	177	193	193	178	190	118	118	130	130	150	160	152	154
MP-57.04	93	133	171	177	193	193	178	190	118	118	130	130	150	160	152	154
MP-57.05	93	133	171	177	193	193	178	190	118	118	130	130	150	160	152	154
MP-57.06	93	133	171	177	193	193	178	190	118	118	130	130	150	160	152	154
MP-59.01	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
MP-59.02	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
MP-59.03	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
MP-59.04	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
MP-59.05	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
MP-59.06	131	131	171	177	181	181	176	200	116	118	124	130	132	160	160	160
MP-62.01	93	135	177	177	191	191	198	200	116	116	130	134	146	158	152	160
MP-62.02	93	135	177	177	191	191	198	200	116	116	130	134	146	158	152	160
MP-62.03	93	135	177	177	191	191	198	200	116	116	130	134	146	158	152	160
MP-62.04	93	135	177	177	191	191	198	200	116	116	130	134	146	158	152	160
MP-62.05	93	135	177	177	191	191	198	200	116	116	130	134	146	158	152	160
MP-62.06	93	135	177	177	191	191	198	200	116	116	130	134	146	158	152	160
MP-62H.01	93	135	177	177	191	191	198	200	116	116	130	134	146	160	152	160
MP-62H.02	93	135	177	177	191	191	198	200	116	116	130	134	146	160	152	160
MP-62H.03	93	135	177	177	191	191	198	200	116	116	130	134	146	160	152	160
MP-62H.04	93	135	177	177	191	191	198	200	116	116	130	134	146	160	152	160
MP-62H.05	93	135	177	177	191	191	198	200	116	116	130	134	146	160	152	160
MP-62H.06	93	135	177	177	191	191	198	200	116	116	130	134	146	160	152	160
MP-68.01	129	131	177	179	187	189	176	200	116	120	120	124	160	162	152	154
MP-68.02	129	131	177	179	187	189	176	200	116	120	120	124	160	162	152	154
MP-68.03	129	131	177	179	187	189	176	200	116	120	120	124	160	162	152	154
MP-68.04	129	131	177	179	187	189	176	200	116	120	120	124	160	162	152	154
MP-68.05	129	131	177	179	187	189	176	200	116	120	120	124	160	162	152	154
MP-68.06	129	131	177	179	187	189	176	200	116	120	120	124	160	162	152	154
MP-78.01	107	131	175	179	177	181	188	200	118	120	120	126	144	200	160	160
MP-78.02	107	131	175	179	177	181	188	200	118	120	120	126	144	200	160	160
MP-78.03	107	131	175	179	177	181	188	200	118	120	120	126	144	200	160	160
MP-78.04	107	131	175	179	177	181	188	200	118	120	120	126	144	200	160	160
MP-78.05	107	131	175	179	177	181	188	200	118	120	120	126	144	200	160	160
MP-78.06	107	131	175	179	177	181	188	200	118	120	120	126	144	200	160	160

Mr-81.01	129	129	177	187	181	191	188	200	116	118	120	130	132	160	154	160
Mr-81.02	129	129	177	187	181	191	188	200	116	118	120	130	132	160	154	160
Mr-81.03	129	129	177	187	181	191	188	200	116	118	120	130	132	160	154	160
Mr-81.04	129	129	177	187	181	191	188	200	116	118	120	130	132	160	154	160
Mr-81.05	129	129	177	187	181	191	188	200	116	118	120	130	132	160	154	160
Mr-81.06	129	129	177	187	181	191	188	200	116	118	120	130	132	160	154	160
Mr-83.01	109	131	175	175	191	191	188	188	120	120	120	130	130	162	160	160
Mr-83.02	109	131	175	175	191	191	188	188	120	120	120	130	130	162	160	160
Mr-83.03	109	131	175	175	191	191	188	188	120	120	120	130	130	162	160	160
Mr-83.04	109	131	175	175	191	191	188	188	120	120	120	130	130	162	160	160
Mr-83.05	109	131	175	175	191	191	188	188	120	120	120	130	130	162	160	160
Mr-83.06	109	131	175	175	191	191	188	188	120	120	120	130	130	162	160	160
Mr-84.01	107	117	175	189	181	191	200	202	116	116	124	130	160	164	150	160
Mr-84.02	107	117	175	189	181	191	200	202	116	116	124	130	160	164	150	160
Mr-84.03	107	117	175	189	181	191	200	202	116	116	124	130	160	164	150	160
Mr-84.04	107	117	175	189	181	191	200	202	116	116	124	130	160	164	150	160
Mr-84.05	107	117	175	189	181	191	200	202	116	116	124	130	160	164	150	160
Mr-84.06	107	117	175	189	181	191	200	202	116	116	124	130	160	164	150	160
Mr-86.01	117	129	175	177	181	191	176	200	116	118	124	130	136	144	152	160
Mr-86.02	117	129	175	177	181	191	176	200	116	118	124	130	136	144	152	160
Mr-86.03	117	129	175	177	181	191	176	200	116	118	124	130	136	144	152	160
Mr-86.04	117	129	175	177	181	191	176	200	116	118	124	130	136	144	152	160
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Mr-86.06	117	129	175	177	181	191	176	200	116	118	124	130	136	144	152	160
Mr-87.01	129	131	171	175	181	181	200	200	118	120	120	130	122	160	152	152
Mr-87.02	129	131	171	175	181	181	200	200	118	120	120	130	122	160	152	152
Mr-87.03	129	131	171	175	181	181	200	200	118	120	120	130	122	160	152	152
Mr-87.04	129	131	171	175	181	181	200	200	118	120	120	130	122	160	152	152
Mr-87.05	129	131	171	175	181	181	200	200	118	120	120	130	122	160	152	152
Mr-87.06	129	131	171	175	181	181	200	200	118	120	120	130	122	160	152	152
Mr-90.01	97	107	171	177	191	191	200	200	120	140	120	120	160	162	152	160
Mr-90.02	97	107	171	177	191	191	200	200	120	140	120	120	160	162	152	160
Mr-90.03	97	107	171	177	191	191	200	200	120	140	120	120	160	162	152	160
Mr-90.04	97	107	171	177	191	191	200	200	120	140	120	120	160	162	152	160
Mr-90.05	97	107	171	177	191	191	200	200	120	140	120	120	160	162	152	160
Mr-90.06	97	107	171	177	191	191	200	200	120	140	120	120	160	162	152	160
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Mr-93.03	131	133	177	187	173	181	176	200	140	140	130	130	150	160	152	160
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Mr-95.01	129	129	171	177	175	191	200	200	116	120	120	130	132	160	154	160
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Mr-95.03	129	129	171	177	175	191	200	200	116	120	120	130	132	160	154	160
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Mr-95.05	129	129	171	177	175	191	200	200	116	120	120	130	132	160	154	160
Mr-95.06	129	129	171	177	175	191	200	200	116	120	120	130	132	160	154	160
Mr-100H.01	111	143	171	177	181	181	188	200	116	140	120	130	160	164	154	160
Mr-100H.02	111	143	171	177	181	181	188	200	116	140	120	130	160	164	154	160
Mr-100H.03	111	143	171	177	181	181	188	200	116	140	120	130	160	164	154	160
Mr-100H.04	111	143	171	177	181	181	188	200	116	140	120	130	160	164	154	160
Mr-100H.05	111	143	171	177	181	181	188	200	116	140	120	130	160	164	154	160
Mr-100H.06	111	143	171	177	181	181	188	200	116	140	120	130	160	164	154	160
Mr-7.01	101	107	177	187	177	181	176	198	116	140	122	122	136	182	154	154
Mr-7.02	101	107	177	187	177	181	176	198	116	140	122	122	136	182	154	154
Mr-7.03	101	107	177	187	177	181	176	198	116	140	122	122	136	182	154	154
Mr-7.04	101	107	177	187	177	181	176	198	116	140	122	122	136	182	154	154
Mr-7.05	101	107	177	187	177	181	176	198	116	140	122	122	136	182	154	154
Mr-7.06	101	107	177	187	177	181	176	198	116	140	122	122	136	182	154	154

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