A Systematic Assessment of Signatures of Positive Selection Events in Natural Populations of the House Mouse

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Bedanken möchte ich mich für die finanzielle Förderung des Maus-Projektes durch die Volkswagen Stiftung.

Zusammenfassung

Frequenz und Struktur von positiven Selektionsereignissen in natürlichen Populationen sind von großer Wichtigkeit, gehören aber zu den noch wenig verstandenen Parametern in der Evolutionsbiologie. Ziel dieser Studie ist die Untersuchung dieser Basisparameter in einem populationsbasierten Ansatz. Positive Selektionsereignisse hinterlassen populationsgenetische Spuren ('Selective Sweeps'), welche in molekularen Studien systematisch identifiziert werden können. Positive Selektion kann zur Fixierung der vorteilhaften Mutation in einer Population führen. Aufgrund des so genannten 'hitchhiking effects', geht die Variabilität in den neutralen, flankierenden Regionen, welche physisch mit dem unter Selektion stehenden Locus verbunden sind, verloren. Diese 'Fußabdrücke' reduzierter neutraler Variabilität können genutzt werden, um systematisch nach positiv selektionierten (adaptiven) Mutationen zu suchen und erlauben eine Abschätzung ihrer Frequenz in einer gegebenen Population.

Unterschiede in der Rekombinationsrate entlang der Chromosomen können die Evolution neutraler Loci durch den 'hitchhiking effect' auch über einen langen Zeitraum beeinflussen. Generell sollten solche Effekte in niedrig rekombinierenden stärker als in hoch rekombinierenden Regionen sein. Detailierte Informationen der physikalischen und genetischen Karte der Hausmaus erlauben es, die Korrelation zwischen neutraler Variabilität und Rekombinationsrate in einer bestimmten chromosomalen Region zu untersuchen. Ich habe Mikrosatelliten aus chromosomalen Regionen unterschiedlicher Rekombinationsraten getestet und innerhalb von fünf beprobten Mauspopulationen (*Mus musculus musculus* and *M. m. domesticus*) keinen Hinweis auf eine Korrelation zwischen Mikrosatellitenvariabilität und Rekombinationsrate gefunden. Das deutet darauf hin, dass die hohe durchschnittliche Mutationsrate von Mikrosatelliten in Säugetieren die Effekte von weitreichendem 'hitchhiking' im Mausgenom ausgleicht.

Analysen, in denen die Variabilität neutraler Marker genutzt wird, um Regionen, die kürzlich unter positiver Selektion standen, zu identifizieren, werden als 'hitchhiking mapping' bezeichnet. Da die Genome hoeherer Eukaryoten ungefähr 40,000 selektierbare Loci enhalten und die Detektion von polymorphen Varianten des Testens von mindestens 20 Individuen aus verschiedenen Populationen bedarf, würde

ein kompletter Genome-Screen eine Bestimmung von Millionen von Genotypen bedeuten. Ich präsentiere hier eine Pool-Strategie, die es erlaubt, die Anzahl an Genotypisierungsreaktionen signifikant zu reduzieren. Die vorgestellte Hochdurchsatzroutine ermöglichte es mir, fast 1000 Mikrosatelliten in verschiedenen Populationen der Hausmaus zu untersuchen. Aus den durch diese Methode produzierten, spezifischen Mustern ist es möglich, visuell die Loci auszuselektieren, die eine populationsspezifische Reduktion in der Varibilität aufweisen. In einem zweiten Schritt werden diese Kandidaten erneut typisiert, diesmal für einzelne Individuen. Die Loci werden statistisch auf Signifikanz überprüft werden.

Eine detailierte Analyse der Kandidatenloci aus einem paarweisen Vergleich zweier Populationen ergab Resultate zur Frequenz von 'Selective Sweeps', der Stärke von Selektion in natürlichen Populationen und der Herkunft der selektierten Varianten. Die zwei untersuchten Populationen der Hausmaus (*M. m. domesticus*) trennten sich vor etwa vor 3000 Jahren nach deren Ankunft in Mitteleuropa. Die massive Invasion von Hausmäusen nach Europa ist, basierend auf fossilen Daten, gut dokumentiert. Starke, unabhängige, nachfolgende 'Bottlenecks' sind wegen den vergleichbaren hohen genom-weiten Variabilitäten in beiden Populationen wenig wahrscheinlich. Aus diesem Grund können komplexe demographische Einflüsse auf die Ergebnisse ausgeschlossen werden. Die identifizierten Kandidatenregionen zwischen diesen beiden Populationen werden durch die Genotypisierung flankierender Mikrosatelliten weitergehend charakterisiert. Die signifikante Abweichung der Kandidatenregionen von dem neutralen Zustand wird durch verschiedene statistische Analysen belegt. Basierend auf diesen Ergebnissen folgere ich, dass es mindestens ein positives Selektionsereignis je 100 Generationen in jeder Linie gegeben haben muss. Da keines der detektieren Sweep-Täler sehr breit ist, im Schnitt sind sie ca. 50 kb, kann gefolgert werden, dass positive Selektion im allgemeinen von Allelen vorangetrieben wird, die einen geringen selektiven Vorteil haben. Errechnete Selektionskoeffizienten variieren zwischen 0,0007 und 0,021. Weiterhin scheint es, dass die vorteilhaften Varianten im Allgemeinen aus der bestehenden Variabiltät hervorgehen und dass positive Selektion als kontinuierlich wirkender Hintergrundeffekt in allen Populationen abläuft.

Abstract

The frequency and the structure of positive selection events in natural populations are of central importance, but one of the least known variables in evolutionary biology. The aim of this study is to investigate these basic parameters in a population based approach. Positive selection events leave population genetical signatures (selective sweeps) behind, which can be systematically identified with molecular studies. Positive selection can lead to the fixation of a favorable mutation in a population. Due to an effect called hitchhiking, variability in the neutral flanking regions which are physically linked to the target of selection is lost. This footprint of reduced neutral variability can be employed to systematically screen for positively selected (adaptive) mutations and allows to estimate their frequency in a given population.

Differences in recombination rates along the chromosomes can influence the evolution of neutral loci via hitchhiking effects even on a large time scale, which would influence the results. Generally, these effects should be stronger in regions of low recombination than in regions of high recombination. The detailed information on physical and genetic maps in the house mouse allows now to assess the correlation between neutral variability and recombination rates at given chromosomal regions. I have tested microsatellite loci from chromosomal regions which show differences in recombination rates and found no evidence for a correlation between microsatellite variability and recombination rates in samples from five wild mice populations (*Mus musculus musculus* and *M. m. domesticus*). This suggests that the high average mutation rate of microsatellites in mammals counter balances the effects of long range hitchhiking in the mouse genome.

Approaches, in which the variability of neutral markers is used to identify regions which have recently been under positive selection, is termed hitchhiking mapping. Since higher eukaryotic genomes may contain about 40,000 selectable loci and the detection of polymorphic variants requires testing of multiple individuals (at least 20) for several populations, a complete genome scan would require millions of genotypes to be determined. I present here a pooling strategy that allows to reduce the number of genotyping reactions significantly. The presented high throughput routine enabled me to investigate almost 1,000 microsatellite loci in different populations of

the house mouse. Among the composite patterns that are obtained in this way, it is possible to visually select those with population specifical reduced variability. In a second step, these candidates were then re-typed in individuals of a carefully chosen population background and statistically tested for significance.

A detailed analysis of the candidate loci identified by a single comparison yield results on the frequency of selective sweeps, the strength of selection acting in natural populations and the origin of selected variants. The two investigated populations of the house mouse (*M. m. domesticus*) have split upon arrival in Middle Europe about 3,000 years ago. The massive invasion of house mice into Europe is well documented based on fossil records. Strong independent subsequent bottlenecks can be ruled out because of a comparable high genome wide variability in both populations. Thus, complex demographic influences on the results can be excluded. Identified candidate regions between the two focal populations were further characterized by genotyping additional microsatellites in the flanking regions of the identified candidates. The significant deviation of the candidate regions from the neutral state is supported by several statistical tests. Based on these results, I find that there was at least one positive selection event per 100 generations in each lineage. Since none of the detected sweep valleys is broad, on average they are about 50 kb, I conclude that positive selection in general is driven by alleles providing weak beneficial impact. Estimated selection coefficients vary between 0.0007 and 0.021. Furthermore, it seems that beneficial variants are generally taken from the standing variation and that positive selection is a continuously acting background effect in all populations.

Declaration

The design of the whole project was done together with Diethard Tautz. Practical laboratory work as well as the major parts of the data analysis was conducted by me. In the different chapters of this thesis I profited from contributions of several co-authors:

Chapter 1

Sonja Ihle introduced me in the laboratory methods, and together with Susanne Krächter she collected ot the mouse samples used in this approach. Some of the microsatellite data was generated by Iary Ravaoarimanana. Thomas Wiehe provided major parts of the background knowledge on data analysis.

Chapter 2

The software pipeline to identify occurrences of dinucleotide repeats in the upstream regions of annotated genes was written by Friedrich Möller together with the help of Thomas Wiehe.

Chapter 3

Diethart and I designed the project together, and the interpretations of all the different results were aquired during numerous discussions. Laboratory work as well as the major parts of data analysis were done by me.

1 General Introduction

1.1 Molecular Evolution – A Short Introduction

Since "All living things have evolved" (Stearns and Hoekstra 2005), a main theme in biology is to understand the processes and mechanisms that facilitate evolutionary change. Irrespective of their function, such changes must have a molecular basis in order to be inherited from generation to generation. Evolutionary changes manifest as mutations in the DNA sequence of single organisms and, at a later stage, these mutations spread in gene pools of populations and species. This study focuses on the appearance and spread of new genetic variants in order to obtain a better insight of the prevalence of these key evolutionary processes.

There are two forces, which drive molecular changes and therefore cause molecular evolution: natural selection and random genetic drift. Drift describes the changes in allele frequency due to the random drawing of gametes that will form the next generation (Hartl and Clark 1997). Thus, drift acts randomly on genetic variation and its impact is larger in smaller populations. In a mutation-drift equilibrium population the probability of fixation is the observed frequency of the allele, and the expected fixation time of new alleles equals four times the effective population size measured in generations.

Natural selection describes all forms of directional changes in the allele frequencies of populations. The direction in these processes is induced from the environment. Two major forms of natural selection are acting in populations: positive selection and negative selection. In practice negative selection is often discussed in the context of background selection (e.g. Nachman 2001), purifying selection (e.g. Hardison 2003, Khaitovich et al. 2004) or selective constraints (e.g. Eyre-Walker et al 2002, Bush and Lahn 2005). In general negative selection refers to the removal of those genetic variants that reduce the fitness of organisms. Positive selection is the force that increases the frequency of beneficial variants and therefore enables adaptive evolution. The fixation time of a favourable allele is not only dependent on the

effective population size but also on the fitness advantage of the beneficial allele. The degree of advantage is usually expressed as the selection coefficient. The value of the selection coefficient describes the decrease in allele frequency of other than the beneficial allele from one generation to the next. If the product of four times the effective population size and selection coefficient is larger than one ($4N_es > 1$) positive selection is acting in a population. Since the relative effect of drift is stronger in small populations, positive selection acts more effectively in large populations. Thus, positive selection events with selection coefficients of e.g. 10^{-3} would be classified as strong positive selection in large populations, like in *Drosophila*, but as weak selection in small populations, like in mice.

Data on the occurrence of positive selection is indispensable in understanding how adaptive molecular evolution acts. Thus, traces of positive selection in genomes are an intensively studies subject (e.g. Harr et al. 2002, Beisswanger et al. 2005). Apart of the gene, which gained a beneficial mutation, the physically linked flanking regions are also affected by the consequences of selection. Together with the beneficial allele of the target gene the flanking regions get a lift in frequency. This effect is called 'hitchhiking effect' (Maynard Smith and Haigh 1974). By the hitchhiking effect the neutral variability in flanking regions is wiped out during the fixation process (see Figure 1.1). Such a positive selection event is termed 'Selective Sweep'. Thus, positive selection leaves characteristic footprints of reduced variability at linked neutral loci in the genome (Maynard Smith and Haigh 1974, Slatkin 1995). The size of the region that is affected by a selective sweep depends on the local recombination rate and the selection coefficient (Maynard Smith and Haigh 1974). The lower the recombination rate and the higher the selection coefficient the larger is the region which is expected to exhibit the footprint of reduced variability (Figure 1.2). In the absence of recombination, hitchhiking eliminates all linked variation. In the presence of recombination, hitchhiking is incomplete since not all variation is removed (Fay and Wu 2000). Following this logic, the often observed positive correlation between levels of nucleotide diversity and recombination rate is widely interpreted as evidence of recurrent selective sweeps (Begun and Aquadro 1993, Nachman 1997, Nachman et al. 1998, Stephan and Langley 1998, Andolfatto and Prezeworski 2001, Betancourt and Presgraves 2002). For loci with very high mutation rates, genetic hitchhiking is expected to produce a correlation between variability

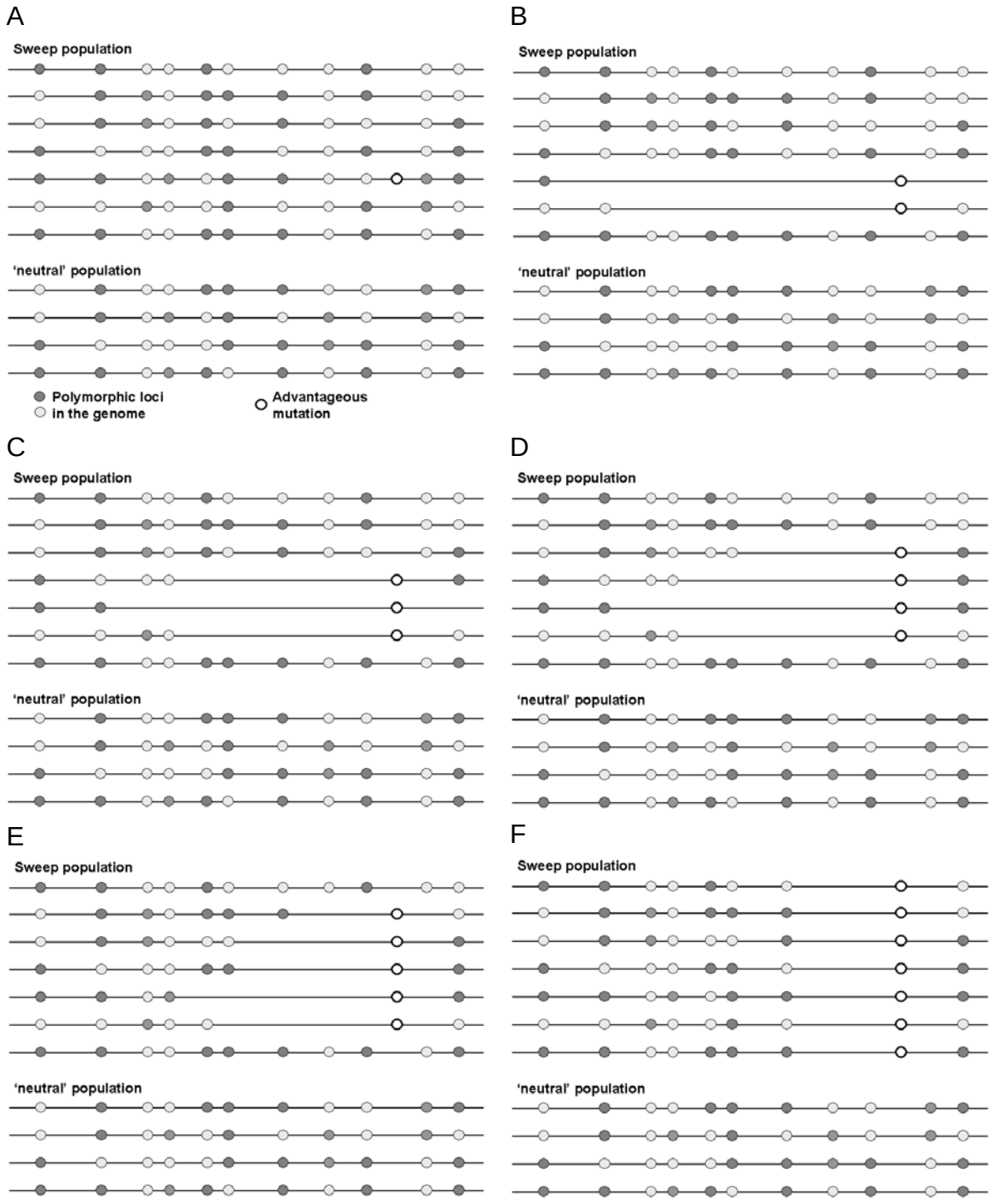

Figure 1.1 The hitchhiking effect. The figure depicts two populations, horizontal lines are chromosomes, the light and dark gray dots represent variable sites. Both populations show many neutral polymorphic sites between the different individuals. If an advantagous mutation appears in one of the two populations (A) this mutation will rise in frequency (C-E). Due to the hitchhiking effect (described by Maynard Smith and Haigh in 1974) the physically linked flanking regions will get a lift in frequency together with the advantageous allele, and thus variability between the individuals is lost in this region. During the time of fixation, the distal flanking regions recover variability because of recombination events. After such a selective sweep, a certain chromosomal area around the gene that carries the beneficial mutation exhibits reduced variability (H). Such reduced variability footprints of positive selection can be identified by comparing variability levels between distinct populations.

and recombination rate only if selective sweeps are very frequent. High mutation rates might counter balance the effects of low recombination rates because of accumulation of new mutations (Slatkin 1995, Wiehe 1998). Knowledge of the correlations between recombination rate and levels of polymorphism in natural populations is essential for projects in which signatures of selective sweeps are used to identify genes experiencing positive selection (Schlötterer 2002). During a selective sweep the increase in frequency of a favourable allele in time follows a sigmoid curve. A beneficial mutation appears in a population and starts at a low frequency. If it does not get lost due to drift, the frequency shows a rapid increase at a certain point in time, until the speed is reduced again before the frequency reaches a plateau, i. e. the allele is fixed. Recombination is expected to take place in the first phase of such a fixation process (Figure 1.3).

With time after the sweep event, the footprint is gradually lost. The recovery pattern is characterized by an excess of new mutations at low frequency. Thus, on the SNP level genetic hitchhiking is expected to produce a skew in the frequency distribution of segregating variants towards an access of rare polymorphisms in the population (Braverman et al. 1995). In microsatellites this recovery pattern can be observed as an increase of new alleles, which occur in a one- or few-repeat-steps distance around the sweep allele.

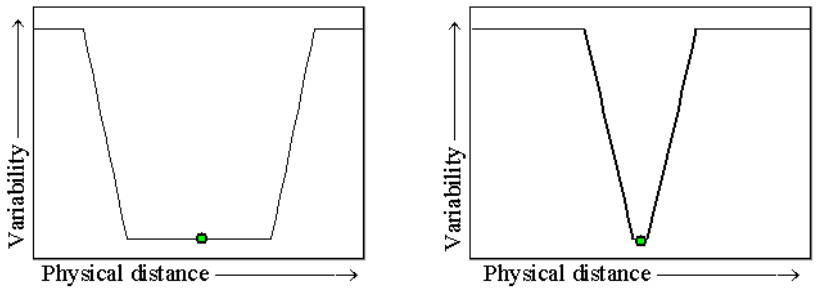

Figure 1.2 The size of the window is dependent on the local recombination rate. The left figure depicts the expected valley in a low recombining area, and the right figure shows a very narrow window, which is expected in a high recombination area for the same selection coefficients.

Screening for signatures of selective sweeps by comparing variability levels between populations is termed ‘hitchhiking mapping’ (Harr et al. 2002, Schlötterer 2003). The timeframe within which such footprints of positive selection are

observable in a hitchhiking mapping approach depends on the mutation rate of the investigated neutral marker. Suitable neutral marker systems for hitchhiking mapping are microsatellites (short tandemly repeated sequences of 1-6 bp in length) and single-nucleotide polymorphisms (SNPs). Although the substitution rates vary between different genomic regions, over the whole genome SNPs follow a more or less constant mutation rate of about 2.5 x 10^{-8} in humans (Nachman and Crowell 2000), 2.1 x 10^{-8} in mice (Nachman 1997), and 3 x 10^{-9} in insects (Andolfatto and Przeworski 2000). Whereas the mutation rate of microsatellites is highly locus specific. It is strongly correlated with the number of repeats a microsatellite carries. The longer a microsatellite, i.e. the more repeats it has, the faster it mutates (see Ellgren 2004 for review). The mutation rate of microsatellites is several orders of magnitude higher than of SNPs. Microsatellites of *Drosophila melanogaster* are short (on average up to 12 repeats) and this lowers their mutation rate. Schlötterer et al. (1998) estimated an average mutation rate of 6.3 x 10^{-6} per locus per generation in flies whereas for the longer microsatellites in humans, mice, rats and pigs mutation rates were estimated in a range of 10^{-2}-10^{-5} (see Schug et al. 1998). Thus, especially in mammals the signatures of selective sweeps are expected to be blurred more quickly by new mutations in microsatellites than on the SNP variability level.

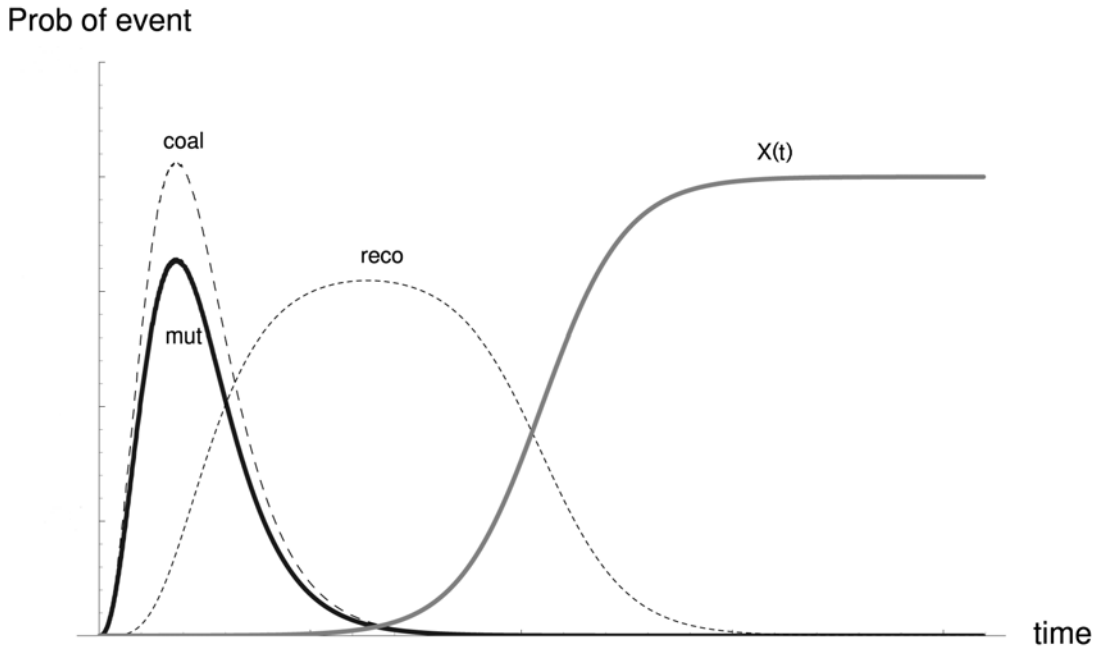

Figure 1.3 The probability that recombination, mutation or coalescence happen during the fixation time of a beneficial allele (Pennings and Hermisson in prep.).

In the genomic era the detection of positive selection on the genome scale is a challenge. Several studies on the theoretical (e.g. Maynard Smith and Haigh 1974, Wiehe 1998), as well as on the comparative genomic level (e.g.Smith and Eyre-Walker 2002, Birne and Eyre-Walker 2004, Bazykin et al. 2004, Keightley et al. 2005) and empirical data from natural populations (e.g. Payseur et al. 2002, Kauer et al. 2003, Kayser et al 2003, Storz et al 2004) have been published. There are two possible types of approaches, first, candidate gene approaches, in which the investigated genes are *a priori* expected to be under positive selection (e.g. resistance evolution (Kohn et al. 2000, Wootton et al. 2002), artificial selection (Vigouroux et al. 2002)), and second, whole genome approaches, where randomly selected genes throughout the genome are investigated. The first kind of approach gives insights into the general pattern of selective sweeps and can provide examples, whereas the second type leads to results concerning the frequency and intensity of positive selection in general. It is still unknown to which degree positive selection acts in nature. Do many genes change a little bit, or do just a few genes undergo great evolutionary changes? This question can only be answered by empirical data from natural populations. The two major model organisms for such population-based approaches are *Drosophila* flies and humans. The focus in experimental studies, so far, is on adaptive changes connected to range expansion (Harr et al. 2002, Kauer et al. 2003, Kayser et al. 2003, Storz et al. 2004). For humans and fruitflies this is the out of Africa event. In this context, again an *a priori* expectation for positive selection is given. Here, selective sweeps are not expected on a certain class of genes but within the derived populations because of large changes in their environmental background. From recently published data by Haddrill et al. (2005) it is known that demographic events can lead to an overestimate of positive selection. Bottlenecks, like the one in *Drosophila* during the out of Africa event can produce reduced variability patterns similar to those of selective sweeps. Thus the differentiation between footprints of positive selection and bottleneck artefacts in regions identified by reduced variability in a derived population compared to an ancestral one is difficult. The selection of a suitable model system for hitchhiking mapping approaches should therefore – beside other important factors – also take care of the demographic background of the populations to be investigated.

1.2 The house mouse

The house mouse has become one of the major model organisms in bio-medical science. It provides many advantages enabling a variety of applications especially in the field of genetics (for review see Guénet and Bonhomme 2003). For several decades the mouse phylogeny and history (e.g. Boursot et al. 1993, Boursot et al. 1996), as well as its behaviour (e.g. Reimer and Petras 1967, Lidicker 1976) was intensively studied. In addition, the complete genome sequence is available since 2002 (Mouse Genome Sequencing Consortium 2002). These data combined provide a perfect background for the study of molecular evolution.

The house mouse evolved on the Indian subcontinent. Less than one million years ago the species *Mus musculus* split into three major sub-species (Figure 1.4). The nominate subspecies *M. m. musculus* which is nowadays found all over northern Asia as well as in Eastern Europe, *M. m. domesticus*, which has its todays range in Western Europe, the Near East, Northern Africa, and recently introduced by humans into the New World, Subsaharan Africa and Australia, and a third subspecies, *M. m. castaneus,* spread all over South East Asia.

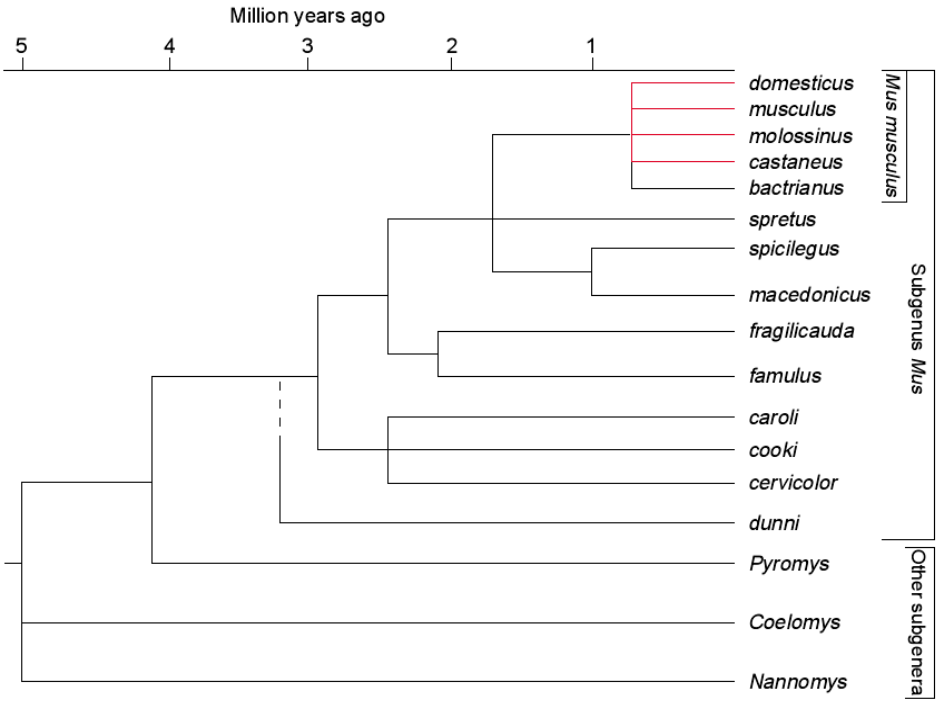

Figure 1.4 Evolutionary tree of the genus *Mus*. The time scale is based on single copy nuclear DNA hybridization studies and is calibrated with the separation of *Mus* and *Rattus*, estimated at 10 Myr ago (taken from Guénet and Bonhomme 2003).

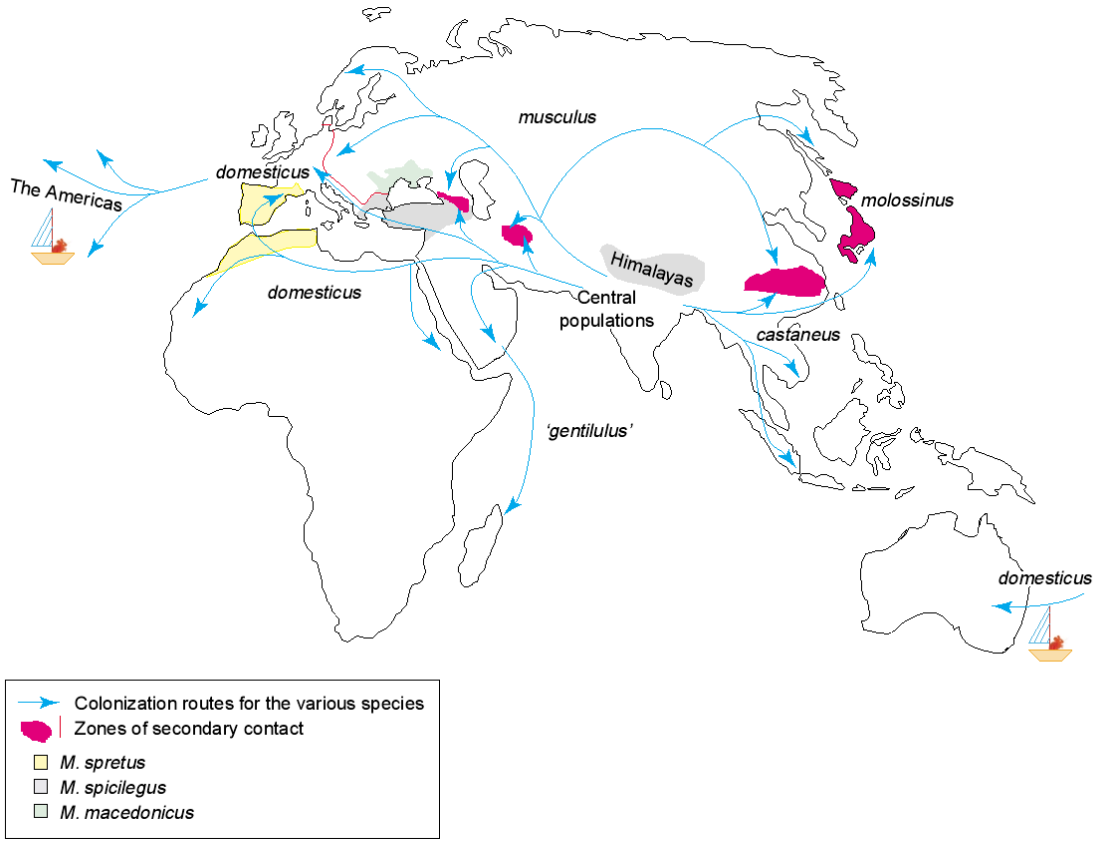

Figure 1.5 Geographical distribution of the different species of the genus *Mus* and routes of colonization. Mice of the American and Australian continents were imported by humans during colonization (taken from Guénet and Bonhomme 2003).

None of these subspecies are completely isolated genetically. There are several natural hybrid zones in the contact areas of their ranges (Figure 1.5). *M. m. musculus* and *M. m. domesticus* meet in Europe, in the Caucasus, and in a region southeast of the Caspian Sea. *M. m. musculus* and *M. m. castaneus* have a contact zone in China. In Japan, these two subspecies have hybridized extensively, giving rise to an unique population often referred to as *M. m. molossinus* (Yonekawa et al. 1988). The two distal subspecies *M. m. domesticus* and *M. m. castaneus* do not have a natural hybrid zone, but they can produce fertile offspring in the laboratory.

The available genome sequence of the house mouse is a mosaic of the three major lineages. About two third of the genome is of '*domesticus*'-origin and the remaining third is of non-*domesticus* origin, i.e. of '*musculus*' and '*castaneus*'- origin (Wade et al. 2002, Wade and Daly 2005). In a recently published paper, Sakai et al. (2005) showed that the '*dometicus*' background of most common laboratory mouse strains (one of which is C57BL/6J, the strain used for the genome sequence assembly)

is mainly derived from the Western European lineage. Thus, the available genome sequence is most similar to mice from these populations.

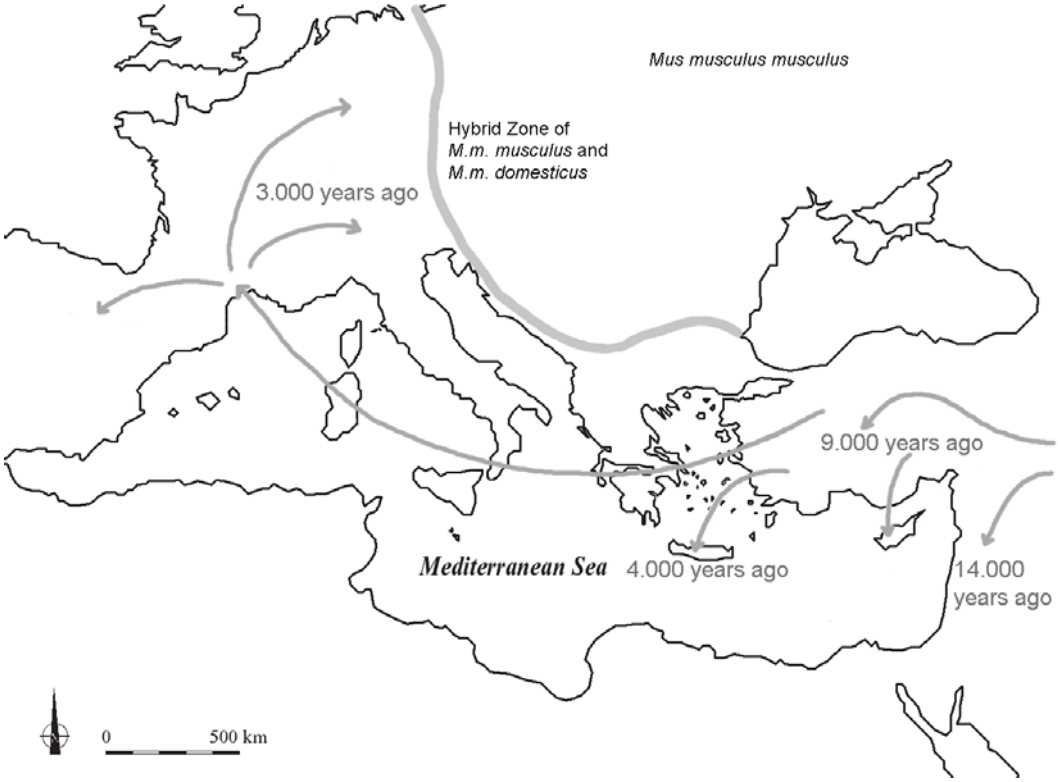

Figure 1.6 Colonization of Middle Europe by the house mouse *Mus musculus domestius* (Figure is based on data by Cucchi et al. (2005)). The mice followed the Neolithic in a massive invasion through Anatolia and then came into Southern France, most likely with Phoenician trading ships, from where they spread all over Western Europe.

The Western European mice have their origin in the Near East. The house mouse, *M. m. domesticus* moved westward from the Near East and entered Europe on the Mediterranean route. Cucchi et al. (2005) published details about the colonization of Western Europe by *M. m. domesticus* based on an analysis of palaeontological records. Their results clearly show that the house mouse invaded Europe not longer than 3,000 years ago via the Near East (in contrast to the route depicted in Figure 1.5 via northern Africa). While humans settled in Europe already 6,000 BC, the mice followed the Neolithic diffusion about 5,000 years later (Figure 1.6). Cucchi et al. (2005) mention three possible reasons for this delayed invasion. First, maritime exchanges were very limited between the Eastern and Western Mediterranean Basin until 1,000 BC, which consequently leads to a very weak migratory flow, probably too weak to maintain stable pioneer populations. Second, the absence of suitable

ecological niches. Since the villages and cities were strikingly different in the early Neolithic, commensal niches were poorly represented and less stable in Western Europe than in the Near East until 1,000 BC. And third, the available commensal niches in Western Europe were favoured by the wood mouse until larger and more stable commensal environments developed. Since the house mouse is not able to live independent of human settlements, especially under non-Mediterranean climate, it was in an inferior position to the wood mouse until the human pressure on the environment increased. After that the house mouse was able to overwhelm the wood mouse in the commensal niches and colonized Europe in its entirety in a massive invasion. Thus, the populations of house mice in Western Europe represent a system which is about 9,000 generations (Karn et al. 2002) old and has its origin in the Near East.

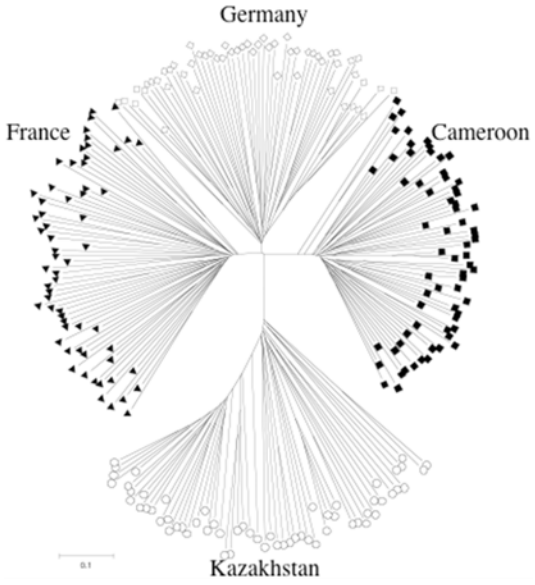

Figure 1.7 Allele sharing tree based on more than 200 microsatellites. The two western European populations, as well as samples from Cameroon can be clearly separated by this data. Samples from a Kazakhstan population, representative of the subspecies *M. m. musculus*, are clearly distinct from the three *M. m. domesticus* populations by a longer branch (Ihle et al. 2005).

The study here is based on samples from natural populations. In the major analysis I focus on two different populations of the Western Eurpean house mouse, one from the Cologne-Bonn-Area and the other one from the Massif Central. Additionally to these two focal samples three other populations were included in different parts of the study, one very young ‚*domesticus*' population from the recently colonized Subsaharan Africa and two populations of the subspecies *M. m. musculus* (one from Kazakhstan, a presumably old population, and one from the Czech Republic). In a previous study we have shown that the investigated populations are all

clearly distict, there is no significant gene flow between them (Figure 1.7, the population from the Czech Republic is not included) (Ihle et al. 2005). Thus, the samples allow the investigation of population specific selective sweeps. In the presence of geneflow between the analyzed poplations, adaptive events would potentially sweep over all populations which have a reasonable exchange.

To summarize, the investigated populations harbour several advantages for the study of evolutionary biology: based on fossil records the history is well documented, the populations are genetically distinct, their maximum diverence time is known, both samples represent derived populations, and a genome sequence, which is in its major parts directly deduced from mice of the same geographical region is avialable.

1.3 Aim of the study

In my study, I am investigating the molecular evolution in natural populations of the house mouse.

- In Chapter 2 I tested and excluded a correlation of recombination rate with microsatellite variability in natural populations. The results suggest that the high average mutation rate of microsatellites in mammals counterbalances the effects of long-range hitchhiking in the mouse genome. Thus, a long term influence of the recombination rate on microsatellite variability can be excluded in mice.

- For systematically genome wide scans for signatures of positive selection a high throughput routine is indispensable. Variability comparisons between natural populations require large amounts of polymorphism data, i.e. an enormous experimental effort has to be performed. In Chapter 3 I introduce a new high throughput routine for genome screens using variability measurements of microsatellites. The routine enables a quick and reliable selection of candidate loci for selective sweeps and therefore reduces the experimental effort to a minimum.

- Applying the described routine I systematically screened the genome for signatures of selective sweeps by comparing variability levels of microsatellites between natural populations. The results enabled me to calculate the minimal frequency with which positive selection events occur in natural populations. In a second step, I studied in detail the structure of the detected footprints of positive selection. The patterns observed in the different regions allowed me to draw conclusions on different basic parameters of selective sweep in general.

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2 Microsatellite variability in wild populations of the house mouse is not influenced by differences in chromosomal recombination rates

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2.1 Abstract

Differences in recombination rates along the chromosomes can influence the evolution of neutral loci via hitchhiking effects. Generally, these effects should be stronger in regions of low recombination than in regions of high recombination. The detailed information on physical and genetic maps in the house mouse allows now to assess the correlation between neutral variability and recombination rates at given chromosomal regions. We have chosen 29 microsatellite loci from chromosomal regions which show differences in recombination rates and have tested their variability in samples from five wild populations of *Mus m. musculus* and *Mus m. domesticus*. Our results provide no evidence for a correlation between microsatellite variability and recombination rates. This suggests that the high average mutation rate of microsatellites in mammals counter balances the effects of long range hitchhiking in the mouse genome.

2.2 Introduction

Levels of polymorphism of neutral loci depend on mutation rate and effective population size. However, neutral loci can be linked to loci experiencing positive or negative selection (see Andolfatto 2001 and Schlötterer 2003 for recent reviews). In this case, parameters such as recombination rate and degree of selection on the linked locus can also influence the level of polymorphism at the neutral locus. For example, a local reduction in polymorphism can provide a signature of a selective sweep, i.e., the recent spread of beneficial mutation (Maynard Smith and Haigh 1974, Braverman et al. 1995, Slatkin 1995). Negative or background selection can also influence the

levels of polymorphism at linked loci (Hudson 1994, Charlesworth et al. 1995; Kim and Stephan 2000). Several cases of correlations between recombination rate and levels of nucleotide diversity are known, and this is widely interpreted as evidence that hitchhiking is common (Begun and Aquadro 1992, Nachman 1997, Stephan and Langley 1998, Nachman et al. 1998, Andolfatto and Prezeworski 2001, Betancourt and Presgraves 2002).

Knowledge of the correlations between recombination rate and levels of polymorphism in natural populations is essential for projects in which signatures of selective sweeps are used to identify genes experiencing positive selection (Schlötterer 2002). We have started a systematic screen for selective sweeps in house mouse using levels of polymorphism of mapped microsatellites (Ihle et al. in preparation). We are therefore particularly interested in whether differences in recombination rate are typically associated with differences in microsatellite polymorphism in mouse. Recombination rates are known to differ across the mouse genome (e.g. Nachman and Churchhill 1996; Froenicke et al. 2002; see Nachman 2002 for review) and detailed genomewide estimates of recombination rates are now available (Rowe et al. 2003, Jensen-Seaman et al. 2004). We have studied here the variability of 29 microstaellite loci in five wild populations and correlate these data with the associated recombination rates. We found no evidence that microsatellites located in regions with different recombination rates had significantly different levels of polymorphism. This result is consistent with findings in humans (Payseur and Nachman 2000).

	Total		*M. m. domesticus*		*M. m. musculus*		Germany		Kazakhstan		France		Cameroon		Czech Republic	
	expHet	VarRep	expHet	VarRep	expHet	VarRep	expHet	VarRep	expHet	VarRep	expHet	VarRep	expHet	VarRep	expHet	VarRep
D1Mit10	0.87	69.81	0.84	69.83	0.4	11.41	0.8	78.38	0.4	11.41	0.85	89.66	0.7	41.3	n.d.	n.d.
D1Mit64	0.84	26.56	0.81	30.3	0.77	11.28	0.78	24.13	0.77	11.28	0.62	17.04	0.38	18.83	n.d.	n.d.
D1Mit70	0.89	55.06	0.89	50.01	0.81	69.56	0.88	62.17	0.81	69.56	0.86	26.27	0.74	55.64	n.d.	n.d.
D1Mit136	0.87	37.7	0.82	16.02	0.89	98.09	0.88	21.9	0.89	98.09	0.83	25.54	0.23	1.43	n.d.	n.d.
D1Mit161	0.87	22.48	0.84	17.4	0.65	7.58	0.8	15	0.65	7.58	0.77	32.15	0.5	1.46	n.d.	n.d.
D1Mit187	0.88	60.34	0.83	17.53	0.76	11.86	0.9	20.29	0.76	11.86	0.79	20.29	0.65	11.84	n.d.	n.d.
D1Mit205	0.9	29.89	0.88	17.98	0.89	43.27	0.88	20.67	0.83	18.25	0.89	23.99	0.75	6.49	0.79	22.57
D1Mit404	0.83	22.56	0.7	6.7	0.9	25.73	0.75	7.63	0.85	23.55	0.6	3.04	0.58	7.8	0.88	11.22
D1Mit456	0.95	1914.94	0.94	377.82	0.79	127.61	0.92	90.6	0.76	16.81	0.96	852.18	0.73	31.98	0.78	241.47
D1Mit512	0.87	41.37	0.86	9.69	0.85	118.48	0.85	7.83	0.85	118.48	0.82	11.73	0.71	6.91	n.d.	n.d.
D2Mit252	0.94	289.71	0.9	154.72	0.91	95.14	0.92	113.31	0.84	82.47	0.78	151.19	0.76	116.93	0.87	14.97
D2Mit383	0.8	6.39	0.79	6.46	0.67	1.64	0.66	2.79	0.44	0.93	0.61	2.09	0.65	11.53	0.65	1.28
D5Mit149	0.91	31.8	0.89	23.05	0.87	26.04	0.86	32.45	0.8	16.87	0.86	26.38	0.63	8.65	0.87	26.22
D5Mit310	0.91	27.05	0.86	10.86	0.87	26.51	0.81	6.51	0.78	15.52	0.87	17.71	0.79	6.68	0.85	26.29
D6Mit180	0.78	51.14	0.52	59.99	0.82	32.63	0.49	63.46	0.73	48.32	0.7	60.51	0.25	41.11	0.71	3.15
D6Mit309	0.88	71.3	0.8	19.17	0.76	38.2	0.86	20.79	0.71	19.2	0.81	20.05	0.3	1.12	0.76	47.47
D7Mit158	0.91	72.47	0.84	47.85	0.87	35.98	0.89	72.25	0.84	20.08	0.68	12.26	0.71	28.22	0.89	50.74
D9Mit61	0.89	38.57	0.9	42.41	0.5	3.91	0.89	114.22	0.5	3.91	0.85	14.14	0.67	29.65	n.d.	n.d.
D9Mit165	0.86	109.18	0.77	10.65	0.85	134.7	0.79	18.17	0.85	134.7	0.73	6.83	0.61	3.41	n.d.	n.d.
D9Mit206	0.89	28.46	0.81	10.22	0.88	56.29	0.83	11.17	0.88	56.29	0.33	6.17	0.73	10.19	n.d.	n.d.
D9Mit223	0.8	10.4	0.74	9.43	0.79	10.04	0.69	7.21	0.82	15.55	0.74	14.19	0.58	1.75	0.7	3.97
D9Mit251	0.92	90.22	0.91	85.3	0.79	28.85	0.86	84.82	0.79	28.85	0.88	30.03	0.72	110.12	n.d.	n.d.
D9Mit330	0.88	66.52	0.79	63.71	0.88	17.36	0.73	70.07	0.82	16.68	0.78	46.98	0.68	75.2	0.84	13.96
D11Mit194	0.92	41.8	0.9	22.07	0.84	32.91	0.9	19.36	0.77	14.44	0.91	23.69	0.65	13.43	0.89	52.43
D11Mit319	0.92	122.62	0.87	126.17	0.89	42.36	0.88	119.18	0.85	34.04	0.86	111.41	0.76	128.88	0.8	50
D13Mit61	0.93	65.33	0.91	32.02	0.91	63.89	0.89	29.39	0.86	29.01	0.88	25.59	0.73	21.96	0.81	98.06
D14Mit67	0.81	99.48	0.56	0.82	0.84	22.42	0.32	0.52	0.75	18.7	0.48	0.51	0.46	0.58	0.86	26.56
D14Mit224	0.91	101.73	0.87	18.14	0.72	13.82	0.84	19.97	0.7	6.83	0.84	19.56	0.77	10.86	0.64	21.3
D19Mit39	0.9	80.94	0.79	37.99	0.95	123.33	0.86	42.39	0.9	72.27	0.79	34.89	0.55	25.42	0.9	26.2
Mean	0.88	127.1	0.82	48.08	0.8	45.89	0.81	41.26	0.77	35.23	0.77	59.52	0.62	28.6	0.8	40.99
standard error	0.01	64.61	0.02	13.54	0.02	7.56	0.02	6.84	0.02	6.57	0.03	28.99	0.03	6.66	0.02	13.04

Table 2.1 Two polymorphism parameters of interest: expected heterozygosity and variance in repeats, shown for all loci for all samples and populations, and summarized for the two subspecies *M. m. musculus* and *M. m. domesticus*.

2.3 Methods

Microstaellite analysis: The following loci were chosen from the mouse database of the Whitehead Institute (http://www-genome.wi.mit.edu/cgi-bin/mouse/index) on the basis of a visual inspection of the recombination maps provided in Rowe et al. (2003): D1Mit64, D1Mit70, D1Mit161, D1Mit10, D1Mit136, D1Mit187, D1Mit205, D1Mit456, D1Mit404, D1Mit512, D2Mit383, D2Mit525, D5Mit149, D5Mit310, D6Mit180, D6Mit309, D7Mit158, D9Mit251, D9Mit61, D9Mit223, D9Mit206, D9Mit330, D9Mit165, D11Mit319, D11Mit194, D13Mit61, D14Mit67, D14Mit224, D19Mit3. Using the recommended primers, the loci were amplified by PCR in 10 µl volumes applying 34 cycles of 45 sec. at 94 °C, 45 sec. at 45 °C, 1:30 min at 72 °C with 9 ng DNA-template, 4 µM primers, 1.5 µM $MgCl_2$, 0.6 µM dNTPs, and 0.4U of Taq-Polymerase. Annealing temperatures were estimated to be optimal between 53 and 62 °C found by applying gradient PCR. The PCR products were diluted in water (diluent volume ranged from 100 to 200 µl). 1 µl of each dilution was run on a 96 capillary sequencer (MegaBACE, Amersham Biosciences) for genotyping. For each run we pooled one FAM, one HEX, and one TET-labelled PCR-product, whereby we always took care that fragments labelled with HEX and TET had a different sizes to avoid problems of interference. We added 0.1 µl ROX 400-Size-Standard and 4.9 µl H_2O per sample as an internal lane standard.

Recombination rates: An estimate of local recombination rate for each locus was obtained by choosing a 10 Mb window centered around the marker. Each of these windows included at least five mapped markers. The genetic positions of these markers were taken from the Whitehead Institute Mouse Genome site (http://www.broad.mit.edu/cgi-bin/mouse/index) and the physical positions were taken from the ensemble genome server (http://www.ensembl.org/Mus_musculus/). The genetic and physical positions were plotted and the slope of the regression-line was taken as the local recombination rate. In addition to our own estimates, we used also the estimates from Jensen-Seaman et al. (2004) for 5Mb and 10Mb nonoverlapping windows.

Statistics: The program Genetic Profiler (Amersham Biosciences, Verson 2.0) was used for the analysis of genotyping data. The program MS-Analyzer (Dieringer and Schlötterer 2003) was used to estimate the expected heterozygosity and the variance of repeats. All parameters of interest were calculated for each population and for the total number of samples. To avoid any interspecific problems this analysis was also done separately for each subspecies. The data were tested by the Kolmogorov-Smirnov-test for normal distribution.

2.4 Results

To obtain polymorphism data from true wild type populations we have sampled mice in five different regions. *M. m. domesticus* populations came from Germany (near Cologne), from France (near Sévérac-le-Château, Massif Central) and from Cameroon (near Kumba). *M. m. musculus* populations came from the Czech Republic (near Námest nad Oslavou) and from Kazakhstan (near Almaty). The sampling scheme took care to avoid related animals which could potentially originate from a single nest, by using only one individual from a particular sampling site. Thus, we consider our samples as representative for the respective local populations.

29 microsatellite loci from ten autosomal chromosomes were chosen to represent different recombination classes. These range from 0.137-1.248 cM/Mb (Figure 2.1). The calculation of these recombination classes is based on using the chosen locus as a center of a 10 Mb window within which the local recombination rate was estimated (see methods). Our rate estimates are generally in agreement with those which were recently provided by Jensen-Seaman et al. (2004) for 10Mb and 5Mb nonoverlapping windows across the genome. Still, since differences exist between the different estimates (Figure 2.1), we have used all three measures for the further analysis.

The 29 loci were typed in the populations for 48 unrelated individuals each. The average values of expected heterozygosity and variance in allele size are depicted in Table 2.1. These measures of polymorphism are all very similar in the populations, with the exception of the Cameroon *M. m. domesticus* population, which showed significantly lower values, possibly due to a bottleneck effect. The large values of average variance in allele size are due mainly to locus D1Mit456, which had 2 classes of allele sizes separated by about 100 bp. We suggest that this bimodality is due to an

insertion in the flanking sequences of the larger group of alleles. A second locus (D14Mit67) showed signs of a selective sweep in the *M. m. domesticus* populations (further details will be presented in Ihle et al., in preparation). These latter two loci were therefore omitted in the further analysis (Table 2.2), although their inclusion would not change the inferences.

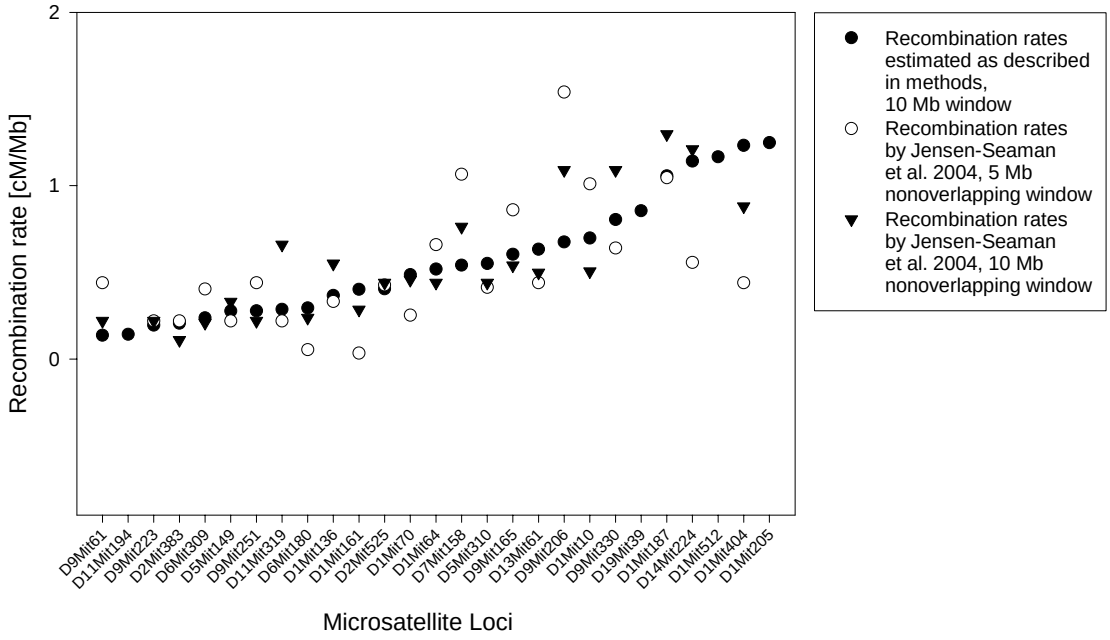

Figure 2.1 Depiction of the loci analysed in relation to their local recombination rate. Three such measures are plotted for each locus. The loci are sorted according to the measure that we have calculated on the basis of a 10Mb window centered around the locus.

Table 2.2 Descriptive statistics of two polymorphism parameters of interest: expected heterozygosity and variance in repeats, without the anomalous loci D1Mit456 and D14Mit67. These values were used for the correlation statistics in Table 3.

Parameter	Statistic	TOTAL	*M. m. domersticus*	*M. m. musculus*	Germany	France	Cameroon	Kazhak-stan	Czech Republic
Expected	N	27	27	27	27	27	27	27	16
Hetero-zygosity	Mean	0.88	0.83	0.80	0.82	0.78	0.62	0.77	0.80
	Standard error of mean	0.01	0.02	0.02	0.02	0.02	0.03	0.03	0.02
Variance in repeats	Mean	61.90	37.62	43.74	40.95	32.35	29.51	36.52	29.36
	Standard error of mean	10.50	7.06	7.46	6.95	6.59	7.08	7.00	6.29

Table 2.2 provides the correlation statistics for the two measures of polymorphism with the three measures of recombination rates. None of the correlations is significant. To correct for the fact that loci with different number of repeats can have different degrees of polymorphism (Goldstein and Clark 1995), we

have also calculated this statistic with normalized values, but find still no significant correlation (Table 2.3).

Table 2.3 Correlation between measure of polymorphism and recombination rate. Three different measures of recombination rate were used. RR1 represents our own calculation with the locus centered in a 10Mb window, RR2 and RR3 are the measures taken from Jensen-Seaman et al. (2004) for 5Mb and 10Mb windows. Note that four of our loci were not included in their estimates, i.e. they were omitted from the analysis.

Samples	Parameter	Statistical test	RR1 N=27	RR2 N=23	RR3 N=23
all populations	Expected Heterozygosity	Pearson's Correlation Coefficient	0.1	0.2	0.3
		p-value (2-tailed)	*0.74*	*0.32*	*0.26*
	normalized values	Pearson's Correlation Coefficient	0.1	-0.1	-0.2
		p-value (2-tailed)	*0.71*	*0.64*	*0.30*
	Variance in Repeats	Pearson's Correlation Coefficient	0.0	0.1	0.1
		p-value (2-tailed)	*0.89*	*0.81*	*0.65*
	normalized values	Pearson's Correlation Coefficient	0.1	0.2	0.1
		p-value (2-tailed)	*0.79*	*0.39*	*0.62*
M. m. domesticus	Expected Heterozygosity	Pearson's Correlation Coefficient	0.0	0.1	0.1
		p-value (2-tailed)	*0.94*	*0.57*	*0.83*
	normalized	Pearson's Correlation Coefficient	0.2	0.2	0.1
		p-value (2-tailed)	*0.42*	*0.32*	*0.79*
	Variance in Repeats	Pearson's Correlation Coefficient	-0.3	-0.1	-0.1
		p-value (2-tailed)	*0.20*	*0.60*	*0.71*
	normalized	Pearson's Correlation Coefficient	-0.1	0.2	0.0
		p-value (2-tailed)	*0.56*	*0.49*	*0.84*
M. m. musculus	Expected Heterozygosity	Pearson's Correlation Coefficient	0.2	0.0	0.3
		p-value (2-tailed)	*0.30*	*0.86*	*0.25*
	normalized	Pearson's Correlation Coefficient	-0.1	-0.2	-0.2
		p-value (2-tailed)	*0.69*	*0.47*	*0.38*
	Variance in Repeats	Pearson's Correlation Coefficient	0.2	0.1	0.0
		p-value (2-tailed)	*0.33*	*0.58*	*0.93*
	normalized	Pearson's Correlation Coefficient	0.3	0.1	0.0
		p-value (2-tailed)	*0.22*	*0.78*	*0.98*

2.5 Discussion

The population sample that was used for this study is unique with respect to representing true wildtype populations of the two known subspecies of the house mouse. Thus, the polymorphism estimates are likely to be very reliable and not influenced by population specific effects. The only exception might be the sample from Cameroon, which represents a population that has only relatively recently colonized this area. Still, even population specific analysis of correlation between polymorphism and recombination rate did not yield significant results in any of the

comparisons (not shown). On the other hand, our result depends on an accurate estimate of local recombination rates. The choice of relatively large windows within which the recombination rates are averaged can potentially confound the presence of recombination hotspots. A simple visual inspection of the recombination maps suggests that such hotspots exist (Figure 2.2). Thus, it is not surprising to see differences in rate estimates, depending on which windows are chosen (Figure 2.1). To compensate for this, we have alternatively tried to classify our markers into high, medium and low recombination groups, by comparing their direct neighborhoods in the map of Rowe et al. (2003). But even such a subjective classification scheme did not yield a correlation between recombination class and polymorphism (not shown). Hence, in spite of the remaining uncertainty of estimating exact local recombination rate, it would appear that our results are robust, at least when taking an average over many loci.

A lack of correlation between local recombination rate and microsatellite variability was also found by Payseur and Nachmann (2000) for humans, in agreement with our mouse data. In contrast to the mammalian studies, Schug et al. (1998) find a positive correlation between microsatellite variability and local recombination rate in *Drosophila melanogaster*. However, microsatellites of *D. melanogaster* are short (on average up to 12 repeats) and this influences their mutation rate. Schlötterer et al. (1998) estimate an average mutation rate of 6.3×10^{-6} per locus per generation whereas for humans, mice, rats, and pigs microsatellite mutation rates were estimated in a range of 10^{-2} to 10^{-5} (see Schug et al. 1997). Slatkin (1995) and Wiehe (1998) have suggested that higher mutation rates will counter balance the effects of selective sweeps in low recombining regions. Thus, high mutation rate potentially explains the lack of association between microsatellite polymorphism and recombination rate in mammals.

Nucleotide substitution rates are much lower than microsatellite mutation rates. The mutation rate per nucleotide in humans is estimated to be 2.5×10^{-8} (Nachman and Crowell 2000), and in mice 2.1×10^{-8} (Nachman 1997). Accordingly, a dependence of nucleotide diversity on recombination rate is evident for both mice and humans (Nachman 1997, Nachman et al. 1998), in line with background selection as well as hitchhiking models. On the other hand, Lercher and Hurst (2002) have suggested that the correlation between nucleotide polymorhism and recombination

rates in humans can be explained by the mutagenic effects of recombination and without invoking the action of selection.

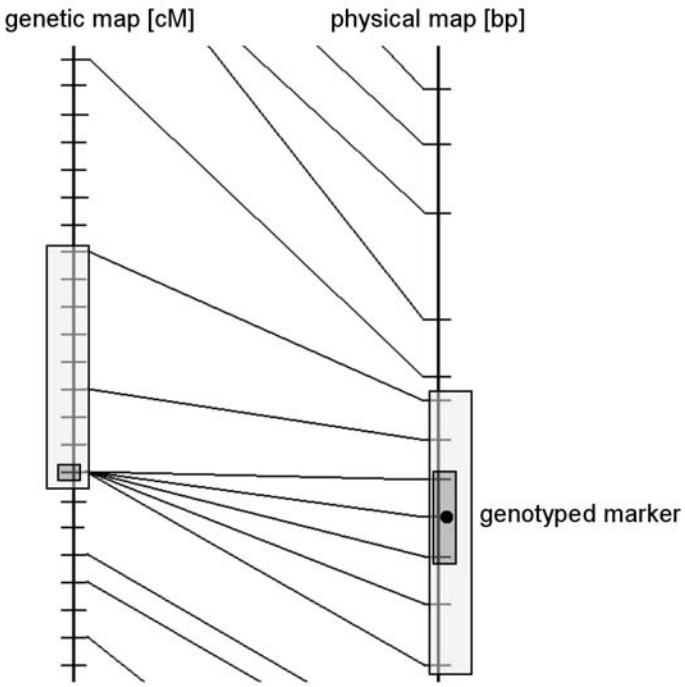

Figure 2.2 Comparison of genetic and physical map to show the problem with averaging recombination rates. Two different window sizes are centered around a marker (shown here for D14Mit224) to estimate recombination rates (light grey about 10 Mb window, dark grey about 5 Mb window). Recombination hotspots can lead to large differences in recombination rate estimates, in dependence of the positioning of the windows.

Furthermore, Hellman et al. (2003) found that not only nucleotide diversity but also divergence rates between human and primates are correlated with recombination rates and that the correlation between diversity and recombination was absent after correcting for divergence. However, recombination rates in humans are highly non-uniform across the genome (McVean et al. 2004) and findings in human may not easily apply to rodents (Hellman et al. 2003). In fact, Jensen-Seaman et al. (2004) reported a negative correlation between diversity and mutation rates in mouse, which may be explained by other genomic properties, such as a different GC-content variation in mouse than in human. It seems therefore possible that also microsatellite mutation rates are influenced by additional genomic factors and that our finding of a lack of correlation with recombination rate is confounded by these additional factors.

It will be the aim of future studies to further disentangle the evolutionary causes which shape microsatellite variability in mice. Still, the current results are compatible with the background selection or hitchking scenarios that take into account mutation rates at microsatellites. However, in mammals microsatellites may be a suitable marker only to detect exceptionally strong selective sweeps, such as the

spread of resistance mutations (Kohn et al. 2000). Theoretical models need to be refined in order to integrate the predictive power of different types of markers, for instance SNPs together with microsatellites, and different measures of variability, for instance heterozygosity (Schloetterer, 2003) together with linkage disequilibrium (Kohn et al. 2003), to get a clearer picture of the regions of the mouse genome under Darwinian selection.

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3 A high-throughput routine to detect signatures of selective sweeps in genome screens using microsatellites

Meike Thomas, Friedrich Möller, Thomas Wiehe and Diethard Tautz

Abstract

We have evaluated a pooling approach that can reduce the number of genotypes in a screen for selective sweeps by more than an order of magnitude. We show that the complex peak pattern that results from pooling of all samples from a given population is a faithful reflection of the composite pattern of the individual alleles, although with an under-representation of the larger alleles. Candidates for selective sweeps can be easily identified by visual inspection of the pool patterns. We have also implemented a software pipeline, which can automatically find suitable microsatellite loci in the vicinity of annotated genes.

3.1 Introduction

Identification of selective sweeps in natural populations has been proposed as an approach to identify genes involved in local adaptations (Schlötterer 2003). Positive selection can lead to the fixation of a favourable mutation in a population. This leads also to a loss of variability in the flanking region, due to hitchhiking (Maynard Smith and Haigh 1974). Because of the high density of microsatellite loci in the eukaryotic genomes it should be possible to trace selective sweeps by systematically scanning for population specific loss of variability at individual loci (Schlötterer 2003). However, since higher eukaryotic genomes may contain about 40,000 selectable loci and the detection of polymorphic variants requires testing of multiple individuals (at least 20) for several populations, a complete genome scan would require millions of genotypes to be determined. We present here a pooling strategy that allows to reduce the number of genotyping reactions significantly and which has been shown to be possible in principle (Pacek et al. 1993).

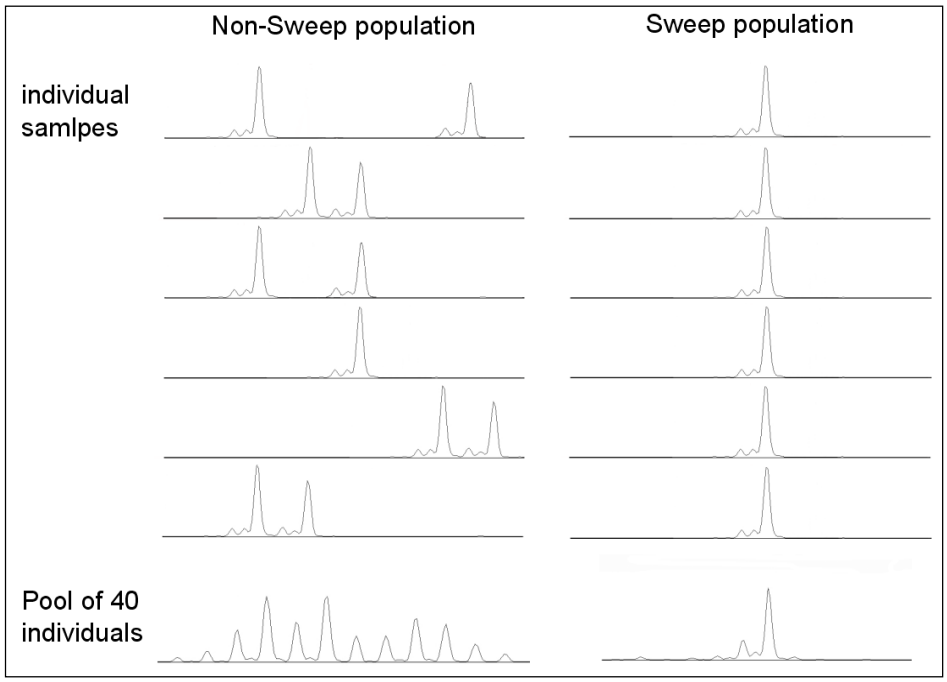

Figure 3.1 In non-sweep populations the pools of individual samples, which are polymorphic, exhibit a complex peak pattern, whereas a sweep locus, which shows the same allele in almost all individuals will show a simple pool pattern.

The basic idea is that DNA samples from all individuals of a given population can be pooled in equal amounts and only one PCR reaction is performed with primers flanking a microsatellite locus. This will result in a complex pattern of peaks in those cases where the locus is polymorphic, but in a relatively simple peak pattern in cases where polymorphism was lost, i.e. in the regions that are candidates for selective sweeps. Such loci can then be re-typed from individuals to confirm the sweep signature and to calculate exact allele frequencies to be used in appropriate statistics. In the following, we describe this approach for a genome screen based on approximately 1,000 microsatellite loci in five different populations of the house mouse (*Mus musculus*).

3.2 The Method

Sample collection schemes and DNA extraction were followed the scheme described in Ihle et al. (2006). DNA of single individuals was normalized to 10 ng/µl and combined in population specific pools, each consisting of 40 samples. Primers were purchased in 96 well plate format (primer sequences see supplementary data)

from Sigma Aldrich. Forward primers were labeled with FAM dye at the 5' end. PCR was performed using a multiplex kit (Quiagen Cat.No. 206143). All reactions were carried out in 10 µl volumes using 30 ng of pooled DNA template and following the protocol of the supplier of the kit. Amplification was started with a 15' melting step at 95°C, followed by 40 cycles of 30'' melting at 94°C, standard annealing temperature for all primers at 60 °C for 1'30'', and elongation at 72°C for 1', followed by a final elongation step at 72 °C for 30'. PCR products were diluted 1:20 in water and 1 µl of this dilution was added to 0.1 µl GeneScan™ 500 LIZ™ Size Standard (Applied Biosystems, Part Number 4322682) in 10 µl HIDI Formamide (Applied Biosystems, Part Number 4311320). Fragments were run on a 48-capillary 3730 DNA sequencer (Applied Biosystems).

The fragment patterns from the pools were displayed with the GeneMapper® Software v3.5 (Applied Bioscience, Part Number 4346647) and then analysed by eye. All output files were inspected by pairwise comparison between populations. Candidate loci were defined as those showing a rather simple pattern of peaks in one population but a complex one in the others. Figure 3.2 shows an example from three loci typed in the five populations, two belonging to the subspecies *Mus musculus musculus* (sampled in Kazakhstan and in the Czech Republic) and three to *M. m. domesticus* (sampled in Germany, France and Cameroon) (Ihle et al. 2006). Because each locus shows characteristic slippage patterns, we have also always included a single individual from each subspecies for comparison.

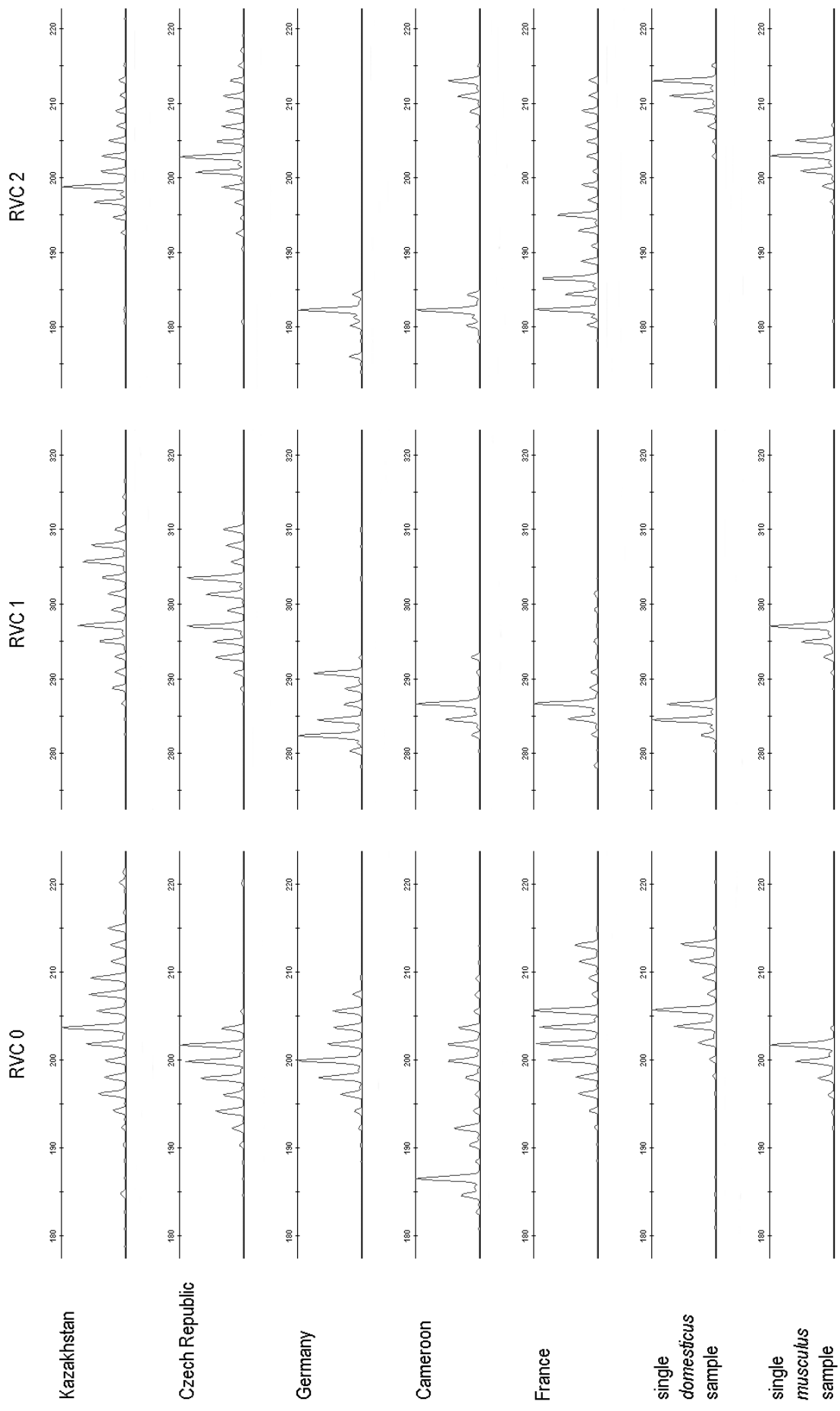
RVC 0
RVC 1
RVC 2
Kazakhstan
Czech Republic
Germany
Cameroon
France
single *domesticus* sample
single *musculus* sample

Figure 3.2 Examples of three output files form the pooling approach each representing one of the three different Reduced Variability Classes. The loci were amplified from all five mouse populations plus a single animal from each subspecies. The classification of the loci in RVC1 and 2 are based on a comparison of the German and the French population. In the example for RVC1 a weak reduction in variability is present in France, but also in Cameroon. The locus classified in RVC2, exhibits almost no variability in Germany compared to a variable French population. With respect to the Cameron population, it would have been classified as RVC1.

3.3 Data Analysis

To classify the differences in reduction in variability, we used a ranking scheme of 'Reduced Variability Classes' (RVC) 0 to 2, where RVC0 contains all loci that show no obvious difference in variability between the populations, RVC1 represents possible cases of reduction in variability and RVC2 represents clear cases. This classification was done by eye, since the complexity of the patterns encountered for the different loci did not allow us to find a simple solution for an automatic quantification, although this may become possible in the future. Figure 3.2 includes examples from each of these ranking classes.

Candidate loci from RVC2 were then amplified from individuals. Figure 3.3 shows three such examples. It is evident that the allele frequencies determined from the individual typings reflect rather well the patterns seen in the pools. However, in several cases we found that larger alleles tend to be under-represented in the pools, when compared to the individual typings. Comparison of the patterns in Figure 3.3 E and F shows that the large alleles in the German population appear only as minor peaks in the pool, but with significant frequencies in the individual typings. This effect has therefore to be kept in mind when evaluating the pool patterns. However, we found no case where an allele appeared with a significant frequency in the individual typings, which was not also at least present as a small peak in the pool pattern. Thus, the pool patterns are fairly reliable indicators of the allele spectrum found in a population.

The results from the individual typings can then be directly used for statistical analysis, for example Schlötterer´s lnRΘ statistic (Schlötterer 2002, Kauer et al. 2003), provided a reference set of randomly chosen loci is available. Using the reference set described in Ihle et al. (2006), we found that the loci in Figure 3.3 A, B and Figure 3.3 C, D show highly significant signatures of selective sweeps, while the locus in Figure 3.3 E, F is not significant (details on this see Chapter 4).

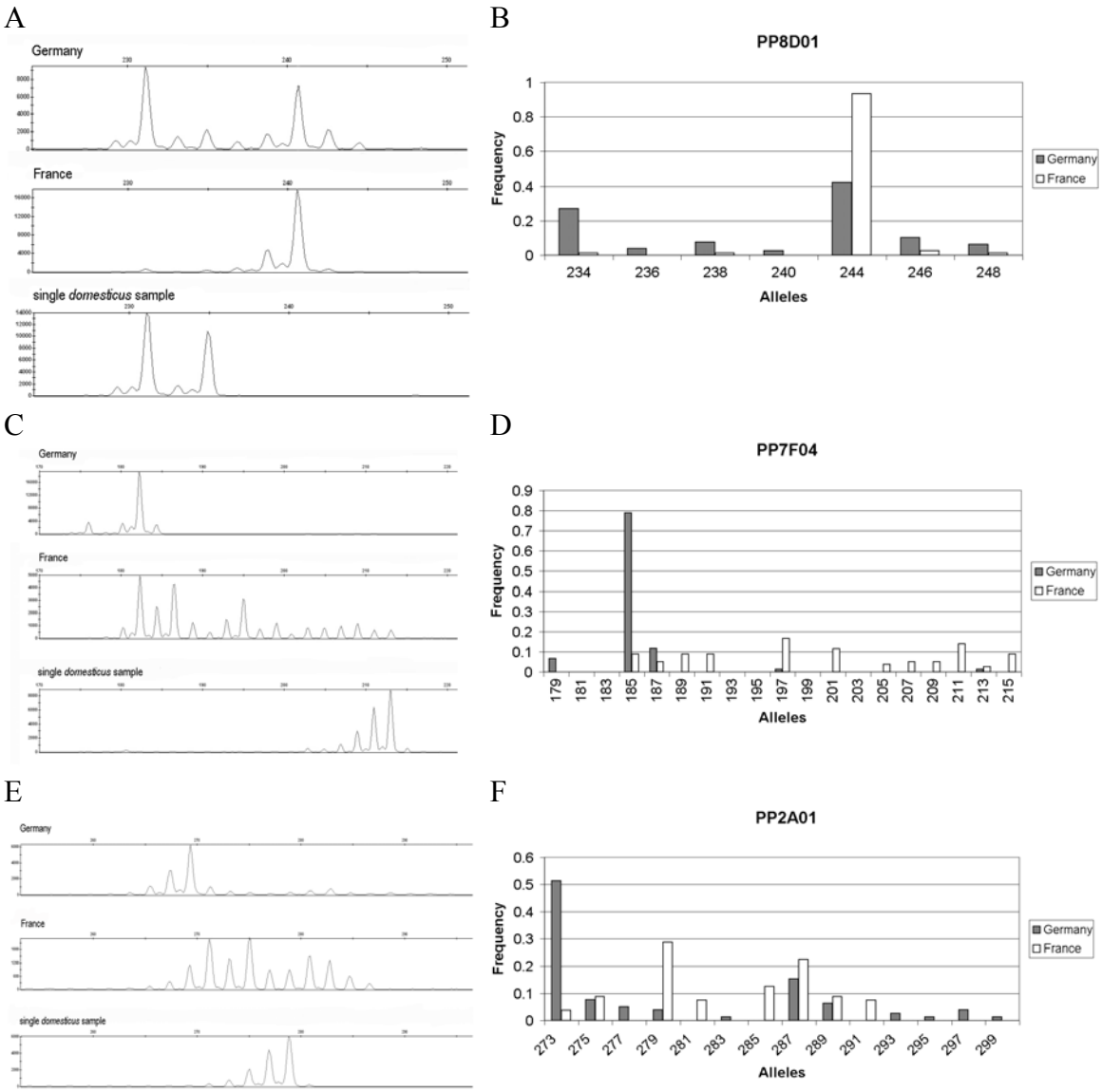

Figure 3.3 Pattern of the pooled samples from three candidate sweep loci (A, C, E) and the corresponding allele frequencies estimated form genotyping single individuals for the respective loci (B, D, F).

3.4 Software Pipeline

Given that the pooling approach allows an efficient screening of large numbers of loci, it is also important to have an automatic routine for selecting the microsatellite loci. Because sweep regions tend to be relatively small (Beisswanger et al. 2005; see Chapter 4) it is advisable to use microsatellite loci from the vicinity of genes for a systematic screen.

To find such loci, we implemented a software pipeline to identify all occurrences of dinucleotide repeats in the upstream region of annotated genes and to

list the results in a comprehensive tabular format. First, the pipeline extracts all upstream regions of annotated genes out of a genbank flatfile. These are then checked against the avialable EST data, to confirm the annotation. The upstream regions of these annotated regions are then extracted, by default 20kb upstream of the putative translation start. In a seed and extend strategy, our pipeline then calls the program ClustDB (Kleffe 2004: http://www.charite.de/molbiol/bioinf/bioinf/Computer programme/ClustDB/clustdb.html), an implementation of a suffix array algorithm. ClustDB finds all repeats of given minimum length in a set of sequences or between two sets of sequences. In our application, we compare the extracted upstream regions with a set of sequences composed of hexamers of all possible dinucleotide combinations. Applying ClustDB to compare these two sequence sets reports all left-maximal occurrences of all dinucleotide repeats on the genomic sequence. In a consecutive step we then (right-)extend the found seeds to their maximum length, and reject those repeats which remain below a given minimum length. Using this positional information, and the genome annotation, which is available from Genbank, the upstream and downstream flanking genes are extracted and returned a tabular output file. The result file contains size range and type of every microsatellite and its physical distance to adjacent upstream and downstream genes. To simplify primer design and further analysis not only the microsatellite sequence itself but also the flanking 600 bp are reported in the output file. This program was used to identify 1,000 loci, distributed across all chromosomes for which primers were designed.

3.5 Conclusions

By applying the pooling strategy we were able to reduce the number of required PCRs from approximately 200,000 to approximately 7,000 for a genome screen involving the previously selected 1,000 loci in five populations, each represented by 40 individuals. In particular, the time consuming part in microsatellite genotyping, namely the allele calling from the raw data, was also reduced to a minimum, because the preselection of candidate loci for signatures of positive selection is based on a simple visual inspection of pool patterns. With our approach it seems therefore feasible to eventually screen all annotated genes of the mouse genome for signatures of selective sweeps.

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4 A Genome Screen gives first Insights into the Basic Parameters and Frequency of Signatures of Selective Sweeps in natural Populations

Meike Thomas and Diethard Tautz

4.1 Summary

One way of identifying the genetic basis of adaptations is to screen genomes for signatures of selection. Positive selection in a genomic region leaves a characteristic footprint behind, namely reduced neutral variation around the selected site (selective sweep). This can be employed to systematically screen for positively selected (adaptive) mutations and allows to estimate their frequency in a given population. However, the key to get a reliable measure of positive selection lies therefore in the choice of populations, for which historical information needs to be available. We have used here a comparison of two populations of the house mouse (*M. m. domesticus*) which have split upon arrival in Middle Europe about 3,000 years ago and for which subsequent major bottlenecks can be excluded. To obtain data on a large number of loci, we have employed a high throughput routine. In a first step, we have pooled the individual samples from natural populations and typed these pools for almost 1,000 microsatellite loci. Among the composite patterns that are obtained in this way, it is possible to visually select those with population-specifically reduced variability. In a second step, these candidates were then re-typed in individuals of a carefully chosen population background and statistically tested for significance. To narrow down the regions potentially affected by positive selection, additional loci in the flanking sequences of the significant candidates were analyzed. All candidate regions clearly indicate valley formations. Based on the size of the regions exhibiting a reduction in variability and the allele frequencies of the candidate loci, we postulate that the majority of positive selection events originates from standing variation and goes along with low selection coefficients as a continuous background effect of natural populations with a minimum frequency of one sweep every 100 generations.

4.2 Introduction

Evolutionary changes are caused by neutral drift and natural selection (Lewontin 1974, Kimura 1983). Despite decades of discussion, the relative importance of these factors is still not clear. One focus of current evolutionary research is to measure the frequency of adaptive changes due to positive selection in natural populations. While the current thinking is dominated by the assumption that neutral evolution shapes the major molecular divergence of genomes (Kimura 1983), Maynard Smith and Haigh (1974) proposed that the frequency of positive selection events may be several orders of magnitude higher than assumed. Until now, most attempts to estimate the average frequency of positive selection in different organisms are based exclusively on comparative sequence analysis between species (Smith and Eyre-Walker 2002, Birne and Eyre-Walker 2004, Bazykin et al. 2004). These are mainly investigations of coding regions to estimate the frequency of adaptive amino acid substitutions. Such comparative sequence analyses are able to measure protein evolution of genes which are under ongoing constraint (e.g. Fay and Wu 2001, Fay et al. 2002) but they miss recent selective events and all adaptive events that go along with changes in cis regulatory systems.

In two recently published studies new methods were presented for revealing functional noncoding DNA from comparative genomic approaches (Chin et al. 2005, Keightley et al 2005)). These projects use sequences of several closely related species and evolutionary theory to estimate the amount of functional regulatory DNA in noncoding sequences. It appears that in the larger genomes of mammals, functional elements are more diffuse than in yeast, but are clustered mostly within 2 kb surrounding protein coding sequences (Castillo-Davis 2005). Gene expression divergence seem to evolve much faster in murids than in humans, and, in comparison to the nucleotide divergence between these two organisms, it is implied that the proportion of gene expression changes that are under natural selection varies between humans and murids. Many of the mutations that affect gene expression in murids may in fact be under positive selection (Keightley et al. 2005). While several authors postulate that regulatory changes play an important role in the evolution of organisms (Zuckerkandl and Pauling 1965, Tautz 2000, Andolfatto 2005, Castillo-Davis 2005), it is obvious that positively adaptive changes in cis regulatory regions can only be measured in the early stages during the presence of selective sweep formations. These footprints are lost over time and distinguishing between single mutations in neutral and regulatory elements in the upstream regions is

difficult.

Previous approaches for identification of selective sweep signatures using microsatellite typing have been applied to situations where positive selection was predicted *a priori*. For example due to colonization of new habitats (Harr et al. 2002, Kauer et al. 2003, Kayser et al. 2003, Storz et al. 2004), resistance evolution (Wootton et al. 2002) or artificial selection (Vigouroux et al. 2005).

Many approaches screening for signatures of positive selection have focused primarily on the invasion of new habitats by humans and *Drosophila*. For both organisms the majority of results confirm the 'out-of-Africa' hypothesis, which predicts more signatures of selection in the derived versus ancestral due to adaptive changes to new environments. However, Haddrill et al. (2005) recently demonstrated that a reasonable number of signatures of positive selection within the derived populations of *Drosophila* are caused by a bottleneck connected to the 'out-of-Africa' event. Thus, the detected difference in the number of selective sweeps in prior studies may be partly due to such artifacts.

For frequency estimations of positive selection events, the major disadvantage of studies on *Drosophila* and humans is the lack of an accurate knowledge of the divergence time. Neither the splitting time of the different *Drosophila* species, nor the dispersal time of *D. melanogaster* populations is exactly known (David and Capy 1988, Lachaise et al. 1988). For *Drosophila,* dates on the 'out-of-Africa' event are anecdotic, and do not provide an accurate time frame (Haddrill et al. 2005). Furthermore, a massive bottleneck followed by an extreme population expansion makes it difficult to distinguish footprints of positive selection from the numerous artifacts (Eyre-Walker 2002).

We have chosen murid populations, which are independent of such *a priori* complications and are sufficiently young to assess the relevant historical parameters. We focus on two populations of the European house mouse *Mus musculus domesticus.* These populations harbour two major advantages: first, a well documented history based on fossil records (Cucchi et al. 2005), and second, the complete genome sequence is available for the mosaic laboratory mouse *Mus musculus* (Mouse Genome Sequencing Consortium 2002). The three major subspecies of the house mouse *Mus musculus musculus*, *M. m. domesticus*, and *M. m. castanaeus* split about 0.5 Mya on the Indian subcontient and from there spread over the world (see Boursot et al. 1993, and Guenet and Bonhomme 2003 for review). Careful analysis of the palaeontological record of

mouse fossils from excavations in human settlements of various ages have shown that the house mouse, *Mus musculus domesticus* invaded Western Europe only about 3,000 years ago from the Near East via the Mediterranean sea (Cucchi et al. 2005). The divergence of populations of the house mouse within western Europe has occurred very recently and therefore footprints of positive selection due to adaptive changes are expected to be still present. Additionally, complex influences of demographic events can be excluded because of the short timeframe within which the populations evolved.

The selection of suitable polymorphic markers and the establishment of a high throughput routine for detecting selective sweeps are necessary for the genomic analysis of several populations (Schlötterer 2002a). Since higher eukaryotic genomes contain roughly 40,000 genes and the empirical detection of polymorphic variants requires tests in multiple individuals (20-50) for diverse populations (2-10), theoretically millions of data points are necessary. Numerous studies have shown microsatellites to be excellent tools for identifying regions influenced by positive selection in a range of organisms (Schlötterer et al. 1997, Huttley et al. 1999, Kohn et al. 2000, Harr et al. 2002, Kauer et al. 2003, Kayser et al. 2003, Payseur et al. 2002, Schlötterer 2002b, Vingouroux et al. 2002, Wootton et al. 2002, Storz et al. 2004). Microsatellites occur at high numbers in most species, and above a certain repeat number they are expected to be polymorphic due to their high mutation rate. Thus, their multiallelic nature makes them informative markers that are particularly well-suited for the characterization of very recent sweeps (Schlötterer 2003, Schlötterer and Wiehe 1999). The complete genome sequence of the house mouse enables us to select and work with a large amount of microsatellites distributed throughout the genome and pinpoint their location with respect to annotated genes.

We sampled five different house mouse populations, three of the subspecies *M. m. domesticus* (one from the Cologne-Bonn area, one from the Massif Central and one from Cameroon), and for resons of comparison two of the subspecies *M. m. musculus* (one from the Czech Republic and one sampled in Kazakhstan). Whereas the major part of the analysis in this chapter is based on the comparison of the two Western Eurpean populations, the German and the Fremch one. To avoid influences of the natural inbreeding behaviour of mice, just one animal from any given sampling site was included in the analysis (Ihle et al. 2006). Genes influenced by positive selection can be identified by the characteristic footprint of reduced variability at linked neutral loci due to a

hitchhiking effect (Maynard Smith and Haigh 1974, Ohta and Kimura 1975, Slatkin 1995). Positive selection increases the frequency of a favourable mutation together with a certain linked neutral region in the population. The size of the region affected by a selective sweep depends on the local recombination rate and the selection coefficient (Maynard Smith and Haigh 1974). After the sweep event, variability is eventually recovered and the footprint is gradually lost by new mutations and random drift. To detect such regions on a genome scale we investigated almost 1,000 microsatellite loci throughout the mouse genome and determined variability levels at these loci in four natural populations. Microsatellites were identified applying a new software tool, which detects dinucleotide stretches within the first twenty kb upstream of start codons of all annotated genes in the genome (see Chapter 3). Investigated microstallite loci were selected randomly, independent of the ontology of the closely positioned gene. To analyze this amount of loci within a reasonable time, we established a novel pooling approach. In this high throughput screening approach we combine the DNA of 40 individuals from each population and amplified all samples in a pooled reaction. This provides a complex pattern of peaks when the locus is polymorphic, but a relatively simple pattern when polymorphism is lost, i.e. when a sweep candidate is detected. Genotyping single individuals shows that the peak-patterns of the pools represent the real allele frequencies reliably (see Chapter 3). The analysis of the pool pattern was done by eye. All output files were inspected by pairwise comparison between populations of the two subspecies. Loci identified by reduced polymorphism in one population but not in others are marked as candidates for selective sweeps.

The results of our investigation allow us to address four basic parameters of positive selection events in natural populations:

- The frequency of positive selection events in the house mouse genome, i.e. how often do such events occur?
- The presence of selective sweeps. Does adaptation always go along with strong environmental changes (like the colonization of new habitats) or is it a continuous background effect in all populations?
- The strength of selection that drives adaptive events. Are selective sweeps driven by strong or by weak selection? That is, do advantageous mutations

in general induce large or small beneficial effects?

- The origin of beneficial variants. Are the beneficial variants taken from standing variation or do adaptive events always go along with new mutations?

4.3 Material and Methods

4.3.1 DNA pooling approach

For the genome screen of 960 microsatellite loci (primer sequences are provided in supplement 1) we used pooled samples as templates. DNA samples of single individuals were normalized to 10 ng/µl and equal volumes of each sample were combined into population-specific pools of 40 individuals each. Primers were ordered from Sigma-Aldrich. Forward primers were labeled with FAM-dye at the 5' end. PCR was performed using a multiplex kit (Qiagen Cat.No. 206143). All reactions were done in 10 µl volumes using 30 ng of pooled DNA template and strictly following the kit protocol. Denaturation at 95°C for 15' was followed by 40 cycles of 30'' melting at 94°C, annealing (for all 960 primers) at 60 °C for 1'30'', and amplification at 72°C for 1', followed by a final elongation step at 72 °C for 30'. PCR products were diluted 1:20 in water and 1 µl of this dilution was added to 0.1 µl 500 (-250) LIZ Size Standard in 10 µl HIDI Formamid. Fragments were run on a ABI 3730 capillary sequencer.

The analysis of the pool pattern was done using Genemapper V.3.5 (Applied Bioscience) to visualize the data. All output files (see digital supplement 1) were inspected by pairwise comparison between the populations of two subspecies. Loci showing a complex pattern of peaks (indicating a certain degree of polymorphism) in one population and a relatively simple pattern (indicating extreme reduction in variability) in the second were marked as candidates for selective sweep (for further details about this pooling approach see chapter 3).

4.3.2 Genotyping single samples

Candidate loci were preselected - with a focus on the comparison of the German and the French population - based on their pool pattern and re-typed for about 40 single individuals of the respective populations. Amplification was done following the

previously described protocol for pooled samples (Chapter 3).

Flanking sequences of 200 Kb surrounding the significant candidate loci were downloaded from Genbank and screened for microsatellite loci applying the program "tandem repeats finder" (Benson 1999). Primers to amplify suitable microsatellites in these flanking sequences were designed with the software "FastPCR" (http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm). All detected loci in these flanking regions were genotyped for the single individuals of the two investigated populations and nucleotide diversity, allele frequencies and lnRH values were calculated.

Furthermore, we downloaded eight regions of 200 Kb randomly taken from 'gene deserts' (visually identified) regions as a neutral control. Each control region came from a different chromosome, with no overlap with the chromosomes of the candidate regions. We screened these 'neutral' regions for microsatellites and amplified all possible loci for single individuals of the German and the French population. LnRH variability along these fragments were calculated.

4.3.3 Statistics

Estimation of significance

Gene diversity estimates (expected heterozygosity, corrected for sample size) for all individually genotyped loci were calculated using the program MS Analyser 3.15 (Dieringer and Schlötterer 2003).

$$\ln RH = \ln \frac{\left(\frac{1}{1 - H(loc1, pop1)}\right)^2 - 1}{\left(\frac{1}{1 - H(loc1, pop2)}\right)^2 - 1}$$

H = heterozygosity
Based on the estimator:
$H = 1 - (1 / (1+2q)^{1/2})$
(Ohta & Kimura 1973)

lnRH statistics (Kauer et al. 2003) was applied to compare variability levels between the populations. This statistic estimates the ratio of variability of a single locus between two populations. Measurement for variability is the expected heterozygosity. The significance of lnRH values is estimated by normalizing the measured values with a dataset of lnRH values from microsatellite loci throughout the genome. lnRH values of this reference data set for normalization should follow a normal distribution. Following a

z-transfomation (z = (x – mean) / standard deviation), a standard normal distribution is approximated and p-values of the investigated candidate loci can be taken from this distribution. In our approach, we used an independent set of 64 'neutrally evolving' microsatellites (collected by Ihle et al. 2006) as a reference to normalize the data. Estimations of mean and standard deviation are based on these 64 loci (mean 0.0875, standard deviation 0.8584); the data does not significantly deviate from a normal distribution (Kolmogorov-Smirnov test: lnRH p=0.724,).

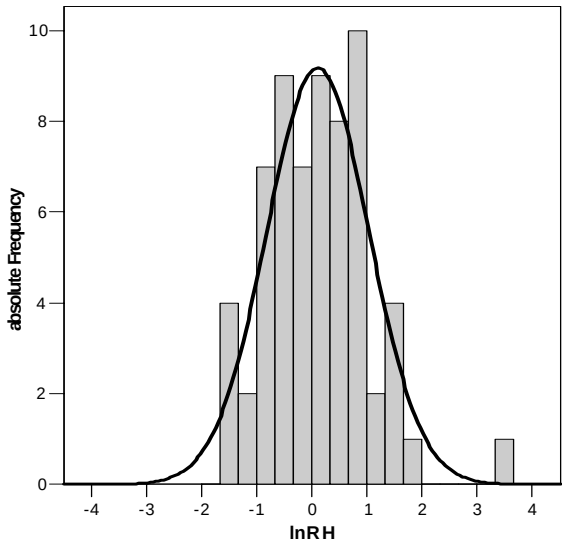


Figure 4.1 Reference data sets. A lnRH reference data set based on 64 'neutrally evolving' microsatellite loci genotyped for individual samples of the two investigated populations, Germany and France.

Significant outliers within the preselected candidate loci were detected by comparing them individually against this data set. Because of the accumulation of false positives due to multiple testing, we followed a stringent Bonferroni-adjustment. Therefore, the significance interval of z-transformed lnRH values ranged from -4.0556 to 4.0556. Significant outliers have p-values smaller than $5 \cdot 10^{-5}$.

Test for deviation of candidate regions from the genome

Deviation of the candidate regions from the neutral regions was studied by comparing the variance of lnRH values between the candidate and the neutral regions. Variance of lnRH was calculated over each of the investigated regions and applying a t-test, these variances were tested for significant differences.

Since demographic events will leave genome wide patterns behind, we included an additional data set of 118 randomly selected microasatellties distributed throughout the

genome (collected by Ihle et al. 2006) taken from the database of the Whitehead Institute/MIT Center for Genome Research and tested the homogeneity of variances in lnRH over all loci of (1) the candidate regions versus the neutral regions (=neutral state), (2) the candidate regions versus the 118 randomly picked loci (=genome variation), and (3) the candidate regions versus the microsatellite loci from the neutral regions together with the 118 randomly picked loci. A Levene test was applied to check for significant differences between the variances within the candidate regions and the variance of the neutral state/the genome variance.

In addition, we applied F-statistics (Weir and Coccerham 1984) to compare differentiation between populations in candidate regions and neutral regions. Population differentiation should be much higher in chromosomal regions that evolved adaptively, compared to neutrally divergent regions. Thus, we calculated global Fst-values over each of the valleys and over each of the investigated neutral regions. These regional global values were tested for significant differences by applying a t-test.

Under a bottleneck scenario one would expect a genome wide influence in the variance of heterozygosity. Thus, we compared the variance in heterozygosity of the 118 randomly selected microasatellties from diverse chromosomes (collected by Ihle et al. 2006) and the microsatellite loci form the neutral regions against the overall variance in heterozygosity of all genotyped loci within the candidate regions applying a Levene test.

In Figure 4.5 and Figure 4.6 z-transformed lnRH values of all investigated loci in the eight candidate and the eight neutral regions are plotted. The shape of the valleys (solid line) is displayed using a sliding window, which includes two to four loci (dependent on the marker density) for each region.

4.3.4 Selection coefficients

The estimation of selection coefficients is based on the heterozygosity values of flanking microsatellites to the most reduced locus and the recombination distance [in Morgans] between them (Stephan et al. 1992).

$$s = \frac{2c \cdot \ln(\frac{1}{2} N_e)}{\ln(1 - y)}$$

s selection coefficient

c recombination distance [recombination rate per bp distance to the sweep locus [bp]]

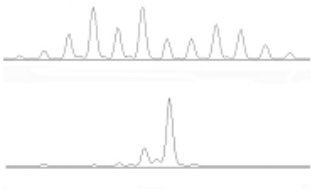
$y = H_1$(flanking locus in the non-reduced population) / H_0 (flanking locus in the reduced population)

Local recombination rates were taken from Jensen-Seaman et al. (2004). Values were calculated independently for each flanking locus. The selection coefficient of each region is an average over all estimated s-values of loci within a valley, i.e. all loci between the sweep locus and the first locus with a lnRH value larger than zero are included.

Flowchart of the approach

GENOME SCREEN

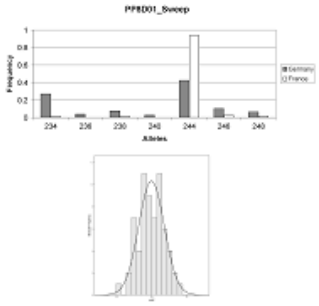
960 pooled loci



Visual inspection of the pool patterns and preselection of candidate loci for further analysis

OUTLIER IDENTIFICATION

40 candidate loci from the comparison of Germany and France



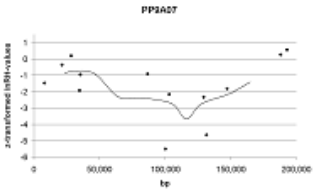
Re-typing of pre-selected candidate loci for single individuals and statistical test for singificance

Independent dataset:

64 loci as reference data set for the lnRθ statistic

VALLEY ANALYSIS

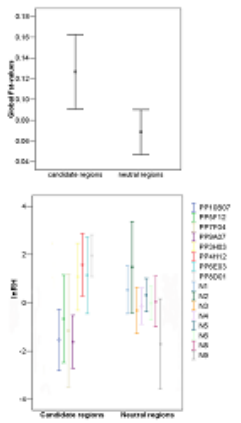
8 significant outlier
4 French sweeps : 4 German sweeps



Selection of microsatellites in 200 kb flanking sequences arround the significant outlier

Selective sweep valleys along the candidate regions

VERIFICATION OF CANDIDATE REGIONS



Statistical analysis applying variance in lnRH and F-statistics for tests of for deviation

Independent datasets:

Neutral regions

118 randomly picked loci throughout the genome

Significant deviation of candidate regions from the neutral state and the genome wide variation

Figure 4.2 Flowchart of the described approach. The selection of candidate loci from the genome screen is based on pairwise comparisons of pool patterns between the German and the French populations. All pre-selected candidate loci are in a second step re-typed for single individuals of the two focal populations. lnRH values of the candidate loci are compared to an independent reference data set of 64 microsatellites to test them for significance. Along 200 kb flanking the eight detected significant outliers, additional microsatellites were genotyped to analyze valley formations of the selective sweeps in these regions. The verification of significant deviations from the neutral state and the genome-wide variation is done by using two additional datasets: One consisting of 118 randomly picked microsatellite loci distributed throughout the genome, which should represent the genome wide variation in lnRH values, and a second one, consisting of microsatellites genotyped in eight neutral regions, which should represent the fluctuations of lnRH values from microsatellites along chromosomal regions of similar size. Applying F-statistics and comparing the variance in lnRH values led to significant results, which verified the deviation and therefore locus-specific evolution of these regions.

4.4 Results

4.4.1 Genome screen applying a DNA-pooling approach

By screening all chromosomes (each represented by 15 to 100 microsatellite loci apart from the Y-Chromosome) in a carefully chosen population background, we were able to detect candidates for signatures of positive selection events on a genome wide scale. Screened microsatellite loci are distributed randomly throughout the genome (Table 4.1). The distance to the start codon of neighboring genes is never larger than 5,000 bp, but in more than 90% of the loci it is less than 2,000 bp.

Table 4.1 Screened microsatellite loci and their distribution over the genome. Preselected loci that were marked for further analysis, and the number of loci that turned out to be significant by lnRH, thus showing a significant signature ($p \leq 5 \cdot 10^{-5}$) of selective sweeps either in the French (Fra) or the German (Ger) populations.

Chromo-some	No. of screened loci	No. of loci classified within RVC2 between Gernamy and France	No. of significant lnRH loci between Germany and France
1	31	4	1 [Fra]
2	66	3	
3	100	3	
4	42	2	
5	49	2	1 [Ger]
6	35	2	
7	83	3	
8	40	1	
9	31	1	1 [Ger]
10	74	1	1 [Fra]
11	83	1	
12	14	1	
13	46	1	1 [Fra]
14	30	2	1 [Ger]
15	37	3	1 [Fra]
16	29	1	
17	35	2	
18	15	1	
19	24	2	1 [Ger]
X	96	5	
	Σ 960	Σ 41	Σ 8 [4 Fra, 4 Ger]

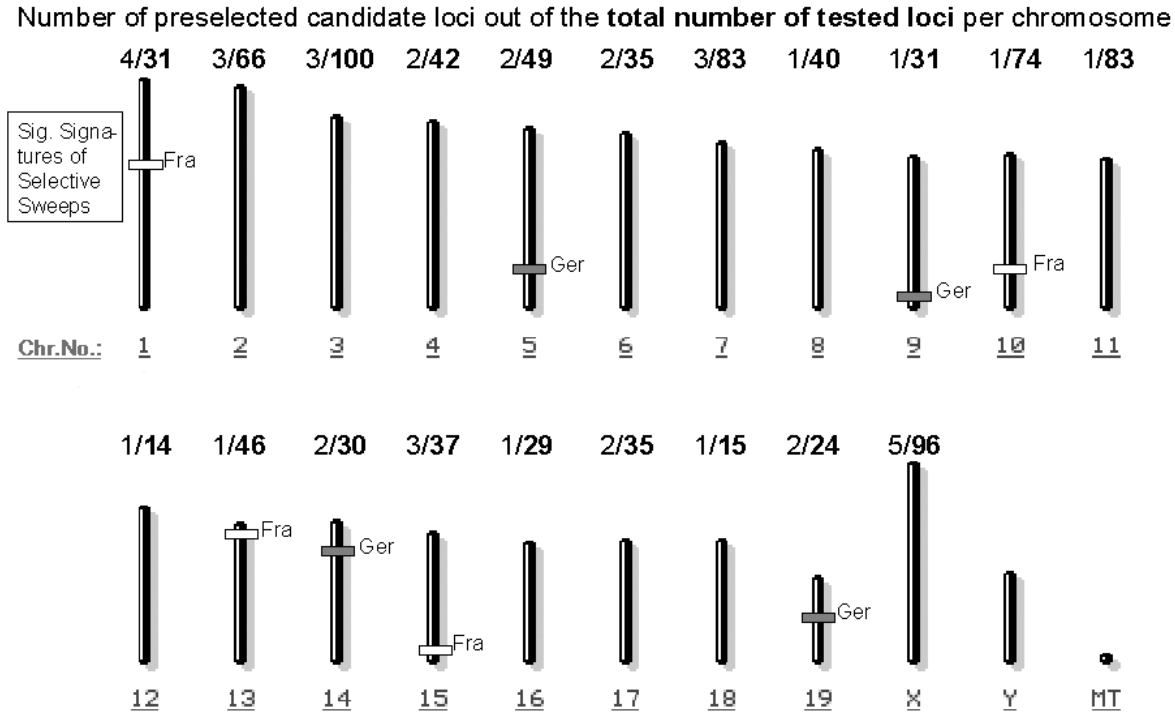


Figure 4.3 Distribution of screened and preselected loci throughout the genome. The location of significant signatures of selective sweeps in the French population are depicted with white and significant signatures in the German population with grey bars.

Almost all of the 960 investigated microsatellites amplified successfully, in only 52 cases were the results inconclusive. By comparing pool patterns of all investigated microsatellites, we found numerous loci of interest for further analysis (for results of the comparison of the German and the French population see Table 4.1).

We marked 41 candidates which showed an extreme difference in variability between the German and the French population. These loci are distributed all over the genome, they are postioned on the autosomes as well as on the X-chromosome (see Figure 4.3). Within these 41 loci, 19 were selected because of an extreme reduction in variability in the German population compared to France, and 22 were selected because of an extreme reduction in variability in the French population compared to Germany. Genotyping single individuals demonstrated that the pool patterns accurately represented the real allele frequencies. Individual typing of loci showing a polymorphic pool pattern in all populations as a control was not required (see Chapter 3).

4.4.2 Verification of candidate loci

To systematically assess the frequency of positive selection events, we genotyped all preselected loci for single individuals of the two focal populations and tested them for

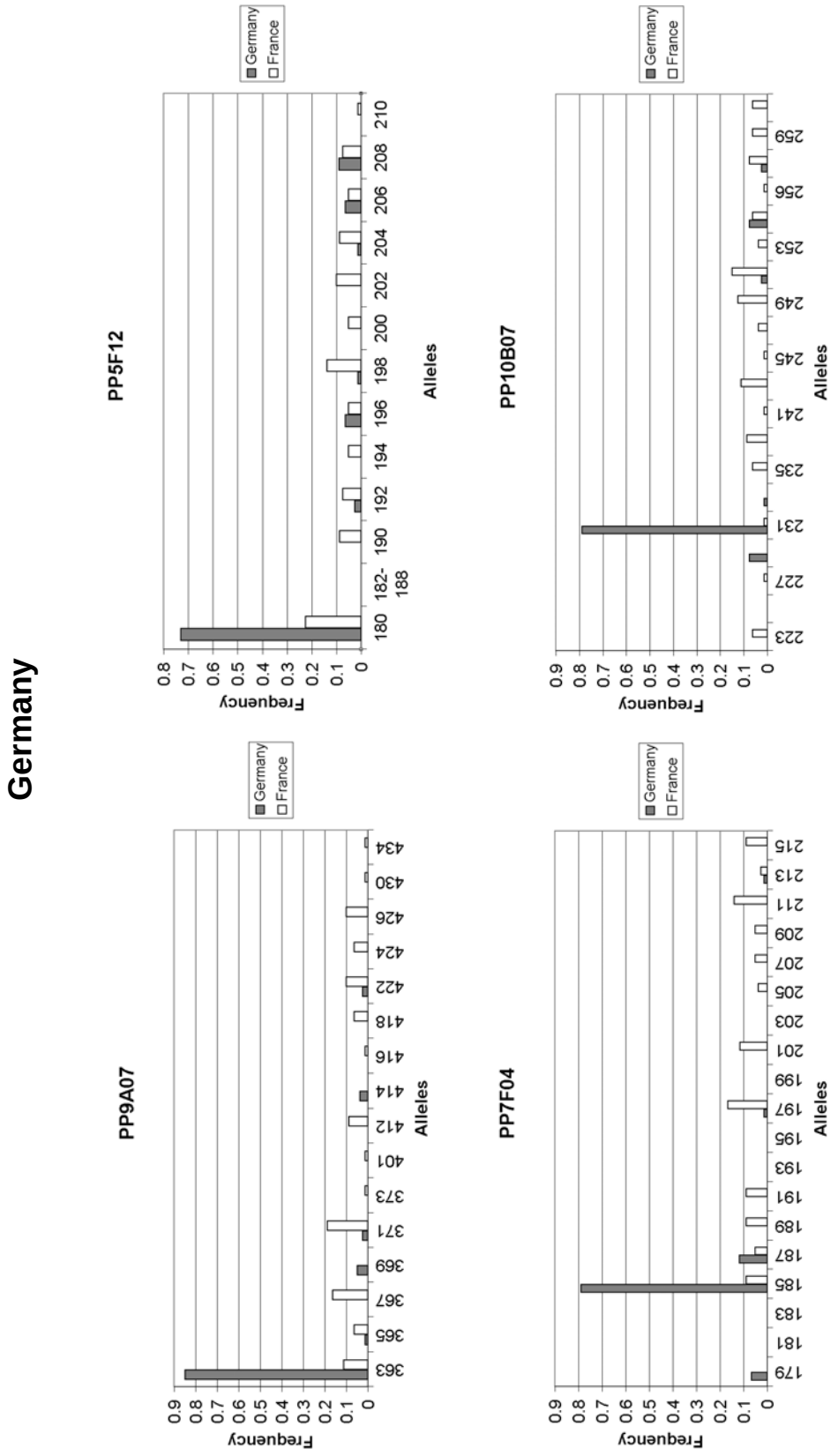
significance. The lnRH statistic (Schlötterer 2002b, Kauer et al. 2003) was applied to identify significant outlier in the comparison of the German and the French population. Candidate loci were individually compared to fixed reference data sets (see Chapter 4.3.3, Ihle et al. 2006). After following a stringent Bonferroni-adjustment, significant outliers have p-values $\leq 5 \cdot 10^{-5}$. According to lnRH, eight of the almost 1,000 screened microsatellite loci showed a significant sweep pattern between the two focal *M. m. domesticus* populations. Significant outliers are positioned on different autosomal regions of the genome and equally distributed between both populations. Candidate loci of the French population were found on Chromosome 1, 10, 13 and 15, whereas loci for the German population were located on Chromosome 5, 9, 14 and 19 (Figure 4.3). Each locus is positioned on a different chromosome, which excludes physical linkage. Recombination rates in these candidate areas vary between 0.22 to 2.06 cM/Mb (Table 4.2 taken from Jensen-Seamen et al. 2004). None of the preselected candidates on the X chromosome showed up as a significant outlier in the applied statistic.

Table 4.2 Loci that showed a significant difference in variability according to the lnRH test statistics, expected Heterozygosities, physical position and recombination rate (taken from Jensen-Seaman et al. 2003)

Marker name	Chromo-some	Physical Position [kb]	Recombination rate [cM/Mb] (5Mb window)	Exp.Het. Ger.	Exp.Het. Fra.	LnRH z-value (p<0.00005)	Sweep in
PP3H03	10	80,316	0.44	0.7092	0.1187	4.1250	Fra
PP4H12	13	3,690	0.22	0.5389	0.0250	4.8689	Fra
PP5F12	19	29,304	1.022	0.4545	0.8949	-4.3378	Ger
PP6E03	1	88,575	0.51	0.7247	0.0737	4.9066	Fra
PP7F04	5	104,783	0.81	0.3628	0.9084	-5.2187	Ger
PP8D01	15	98,140	2.06	0.7353	0.1277	4.2583	Fra
PP9A07	14	43,925	0.44	0.2756	0.8968	-5.4968	Ger
PP10B07	9	109,088	0.342	0.3718	0.9244	-5.6120	Ger

In almost all cases one of the shortest alleles is fixed. Only for locus PP8D01, the second longest allele is the major one in the sweep population. All French sweep loci showed a sweep allele frequency of more than 90%, whereas in three of the German candidates the sweep allele frequency was below 90%. In two of these more than 80% of the chromosomes carried the major allele and at the third locus the allele is present in more than 70% of the samples. In all cases the sweep allele is also present in the non-sweep population (see Figure 4.4).

Based on the pool patterns we found two candidates, for which we can be reasonably shure that lnRH statistic would yield significant results for a population specific sweep in Kazakhstan compared to the samples collected in the Czech Republic (both belong to the subspecies *M. m. musculus*). The samples from Kazakhstan represent an ancestral population (data not shown).


Germany
PP5F12
PP10B07
PP9A07
PP7F04
Germany
France
Frequency
Alleles

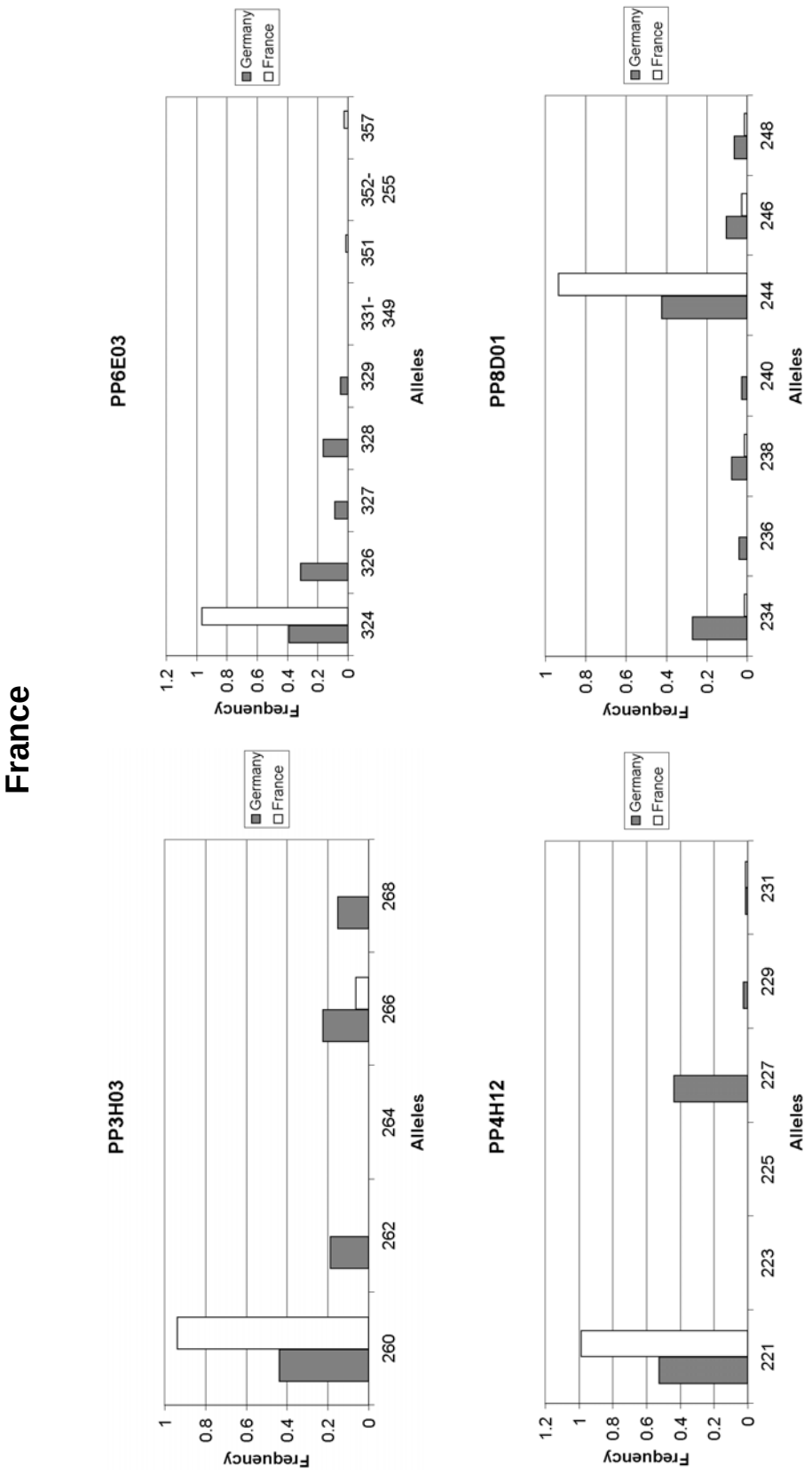


Figure 4.4 Allele frequencies of the eight significant outlier loci. In all cases at least four alleles were present in the variable population, and beside the one major allele in the sweep population, always one or more low frequency alleles were detected which appear distal to the high frequency allele.

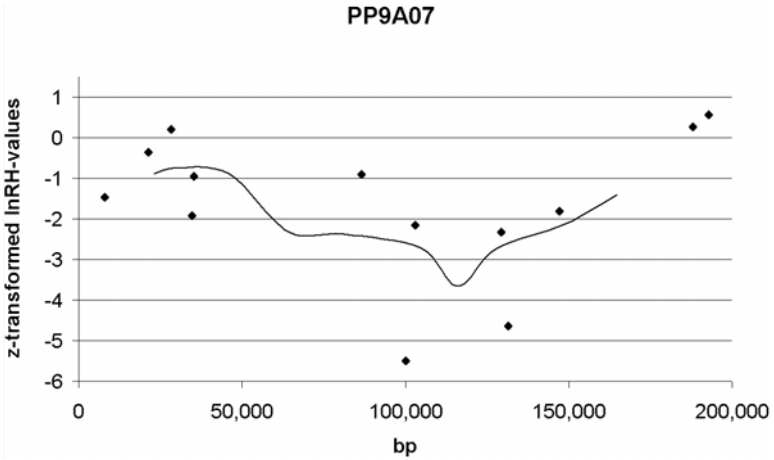
4.4.3 Sweep valley analysis

The size of the sweep window depends on the strength of selection and the local recombination rate. If the former is high and the latter is low, we would expect relatively large regions with reduced polymorphism. To assess the size of the sweep windows, we have typed flanking microsatellites for each locus with a significant sweep signature (primer sequences of these flanking microsatellites see supplement 3). We covered areas between 60 and 180 Kb surrounding the preselected outlier with five to twelve neighbouring microsatellite loci (see Figure 4.5). To cover the candidate regions as densely as possible we selected not only dinucleotide repeats, but also tri- and tetranucleotide repeats.

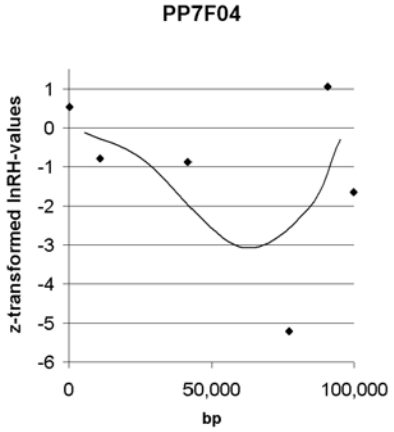
Testing whether the fluctuation of microsatellite lnRH values within these candidate regions deviates from the fluctuations of neutrally evolving regions requires a comparison to such regions. Thus, we picked eight areas on different chromosomes (Chromosome 3, 4, 6, 7, 8, 11, 12, and 17). We took two factors into account when selecting these areas: First, we picked them in 'gene-deserts' regions (visually identified) to reduce potential influences of selection, and second, chose loci that do not reside in regions with extreme recombination rates (Jensen-Seaman et al. 2004). Microsatellite loci along these eight regions were genotyped. Areas of 80 to 180 Kb were covered with numerous microsatellites (three to nine per region) (Figure 4.6).

Compared to lnRH fluctuations of microsatellites in neutral regions, four (PP8D01, PP10B07, PP4H12, PP9A07) of the eight candidate regions clearly indicate valley formations around the preselected microsatellites. These regions are positioned on Chromosome 9, 13, 14, and 15, two exhibiting a reduction in variability in the German population and two in the French population. Three of the four regions contain at least one closely positioned microsatellite flanking the preselected locus significantly reduced at the 5% level. The fourth locus (PP10B07) is flanked by two mirosatellites without a significant lnRH value. However, in this case, in both directions the second flanking microsatellites are significantly reduced at the 5% level.

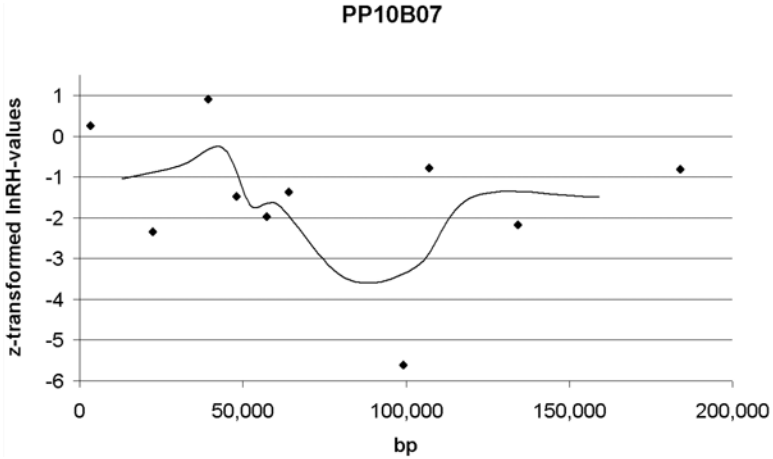
Germany



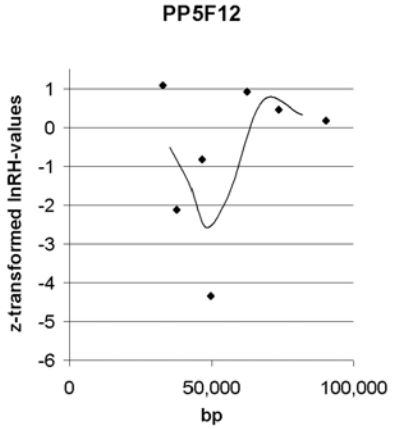
Chr. 14 / pp. 43,925 kb / r = 0. 44 / s = 0.0018



Chr. 5 / pp. 104,140 kb / r =0.81 / s = 0.0029

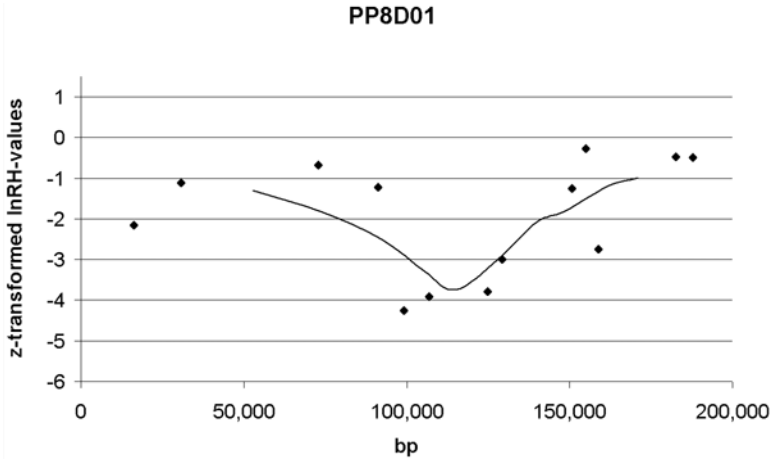


Chr. 9 / pp. 109,130 kb / r = 0.34 / s = 0.0015

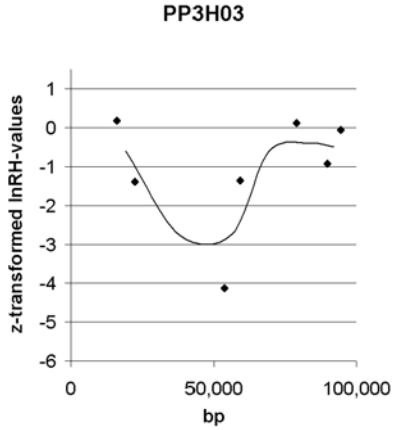


Chr. 19 / pp. 29,304 kb / r = 1.0 / s = 0.0011

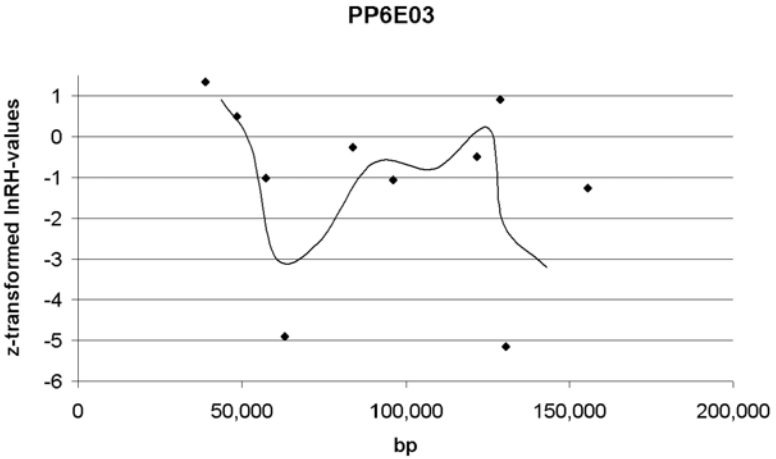
France



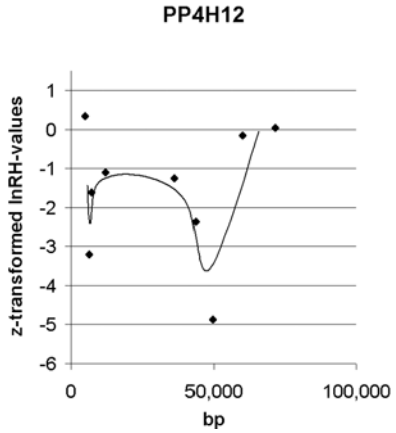
Chr. 15 / pp. 98,140 kb / r = 2.06 / s = 0.021



Chr. 10 / pp. 80,540 kb / r = 0.44 / s = 0.0008



Chr. 1 / pp. 88,575 kb / r = 0.51 / s = 0.0013

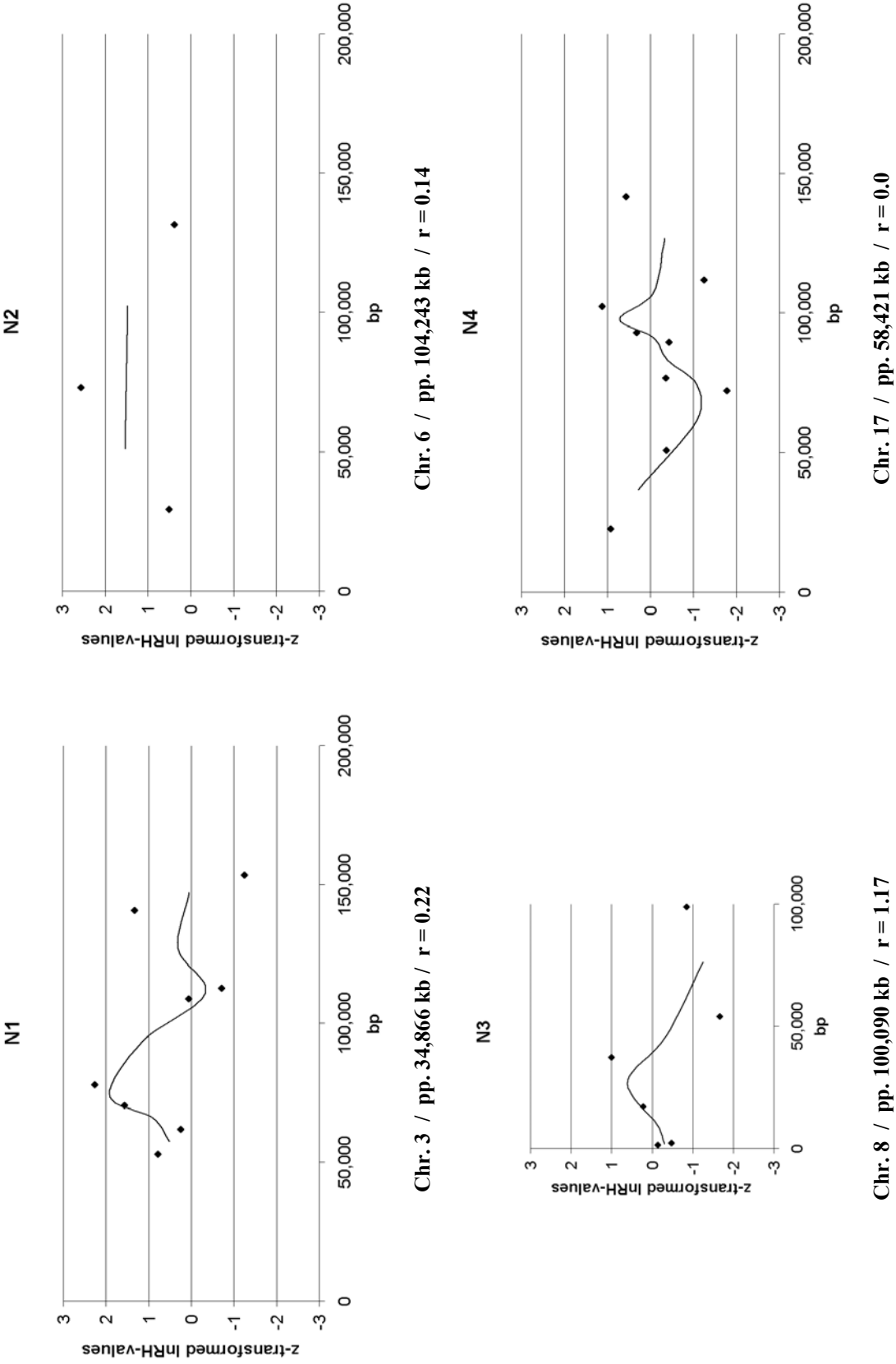


Chr. 13 / pp. 3,690 kb / r = 0. 22/ s = 0.0007

Figure 4.5 Depiction of sweep valleys around the loci with highly significant signatures of selective sweeps. The four loci in the left column represent the loci found in the German population, the ones in the right column the French population. Normalized (z-transformed) lnRH values are plotted against chromosomal position. All graphs have the same scales. lnRH values below 4.05 are highly significant ($p < 5 \cdot 10^{-5}$). The top four regions show at least two highly significant sweep loci within the valley. The lines represent the averages of the lnRH values, as determined by a sliding window technique (see Methods). "pp." represents the position in the genome sequence of the respective chromosome, "r" represents the local recombination rate, "s" the estimated selection coefficient (see Methods). Note that the lnRH values are not always consistently low in a given valley. We ascribe this to different mutation rates at different loci, or for different alleles, i.e. although all loci in a valley are expected to have gone through the sweep, some would recover more quickly than others because of a high primary mutation rate (see also supplement material).

The other four investigated regions (PP6E03, PP5F12, PP3H03, PP7F04), do not contain flanking loci exhibiting significant reduced variability. Here, the lnRH-values fluctuate diffusely. The lack of significant reduced variability in the flanking regions might be due to the absence of closer positioned microsatellites. These results are consistent with the observations of Harr et al. (2002), Kauer et al. (2003) and Kayser et al. (2003), who observed several regions for which the reduction in variability could be confirmed by flanking loci, but others lack significance in the flanking regions.

In Figure 4.7 the allele frequencies of microsatellites genotyped within two candidate regions are illustrated. The number of alleles varies between the investigated microsatellite loci. Given that the same number of individuals is always typed, a high number of alleles is an indication of a large number of repeats (since the number of detected alleles strongly correlates with the sample size). The more repeats a locus has, the faster it mutates. Thus, microsatellites with high mutation rates already might have recovered variability, whereas others with lower mutation rates, might still show the extreme reduction in variability due to the sweep event. This might be also the reason for the often observed pattern of the shortest allele being the sweep allele. Loci in which another than the shortest allele went to fixation due to a selective sweep event recover variability faster, because longer alleles have higher mutation rates than shorter alleles. If only one very short allele is present in a population, it might take a longer time to accumulate new alleles via mutations than in situations where a long allele went to fixation by positive selection. This gives us a possible explanation why we observe this pattern in almost all candidates. The sweep pattern persist longer, and is therefore more likely to be detected, in short fixed alleles, and is faster blurred in longer ones. The regions influenced by significantly reduced variability ranged from 20 kb to more

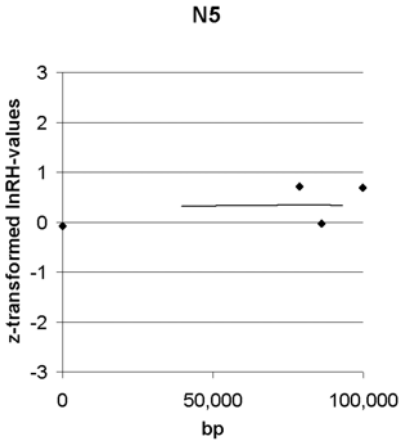


Chr. 3 / pp. 34,866 kb / r = 0.22

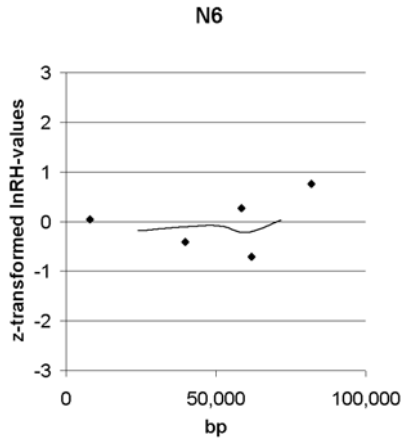
Chr. 6 / pp. 104,243 kb / r = 0.14

Chr. 8 / pp. 100,090 kb / r = 1.17

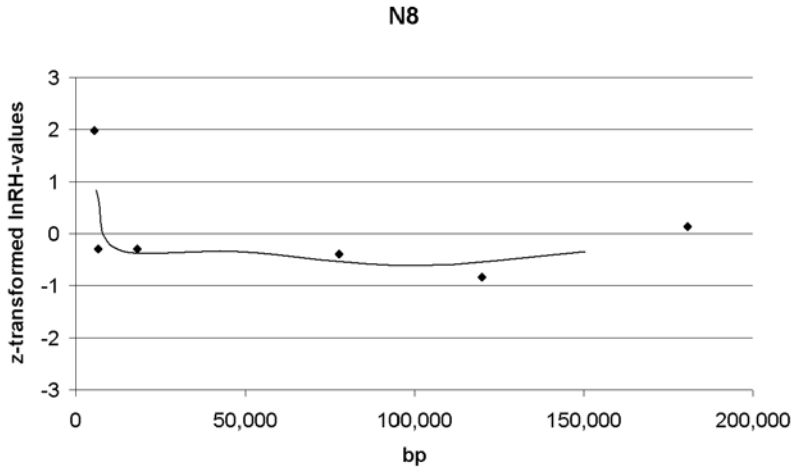
Chr. 17 / pp. 58,421 kb / r = 0.0



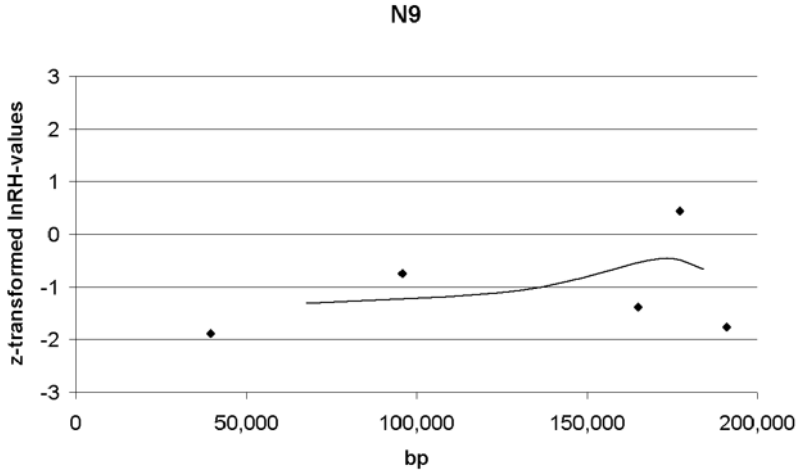
Chr. 4 / pp. 27,402 kb / r = 0.0



Chr. 12 / pp. 92,724 kb / r = 0.40

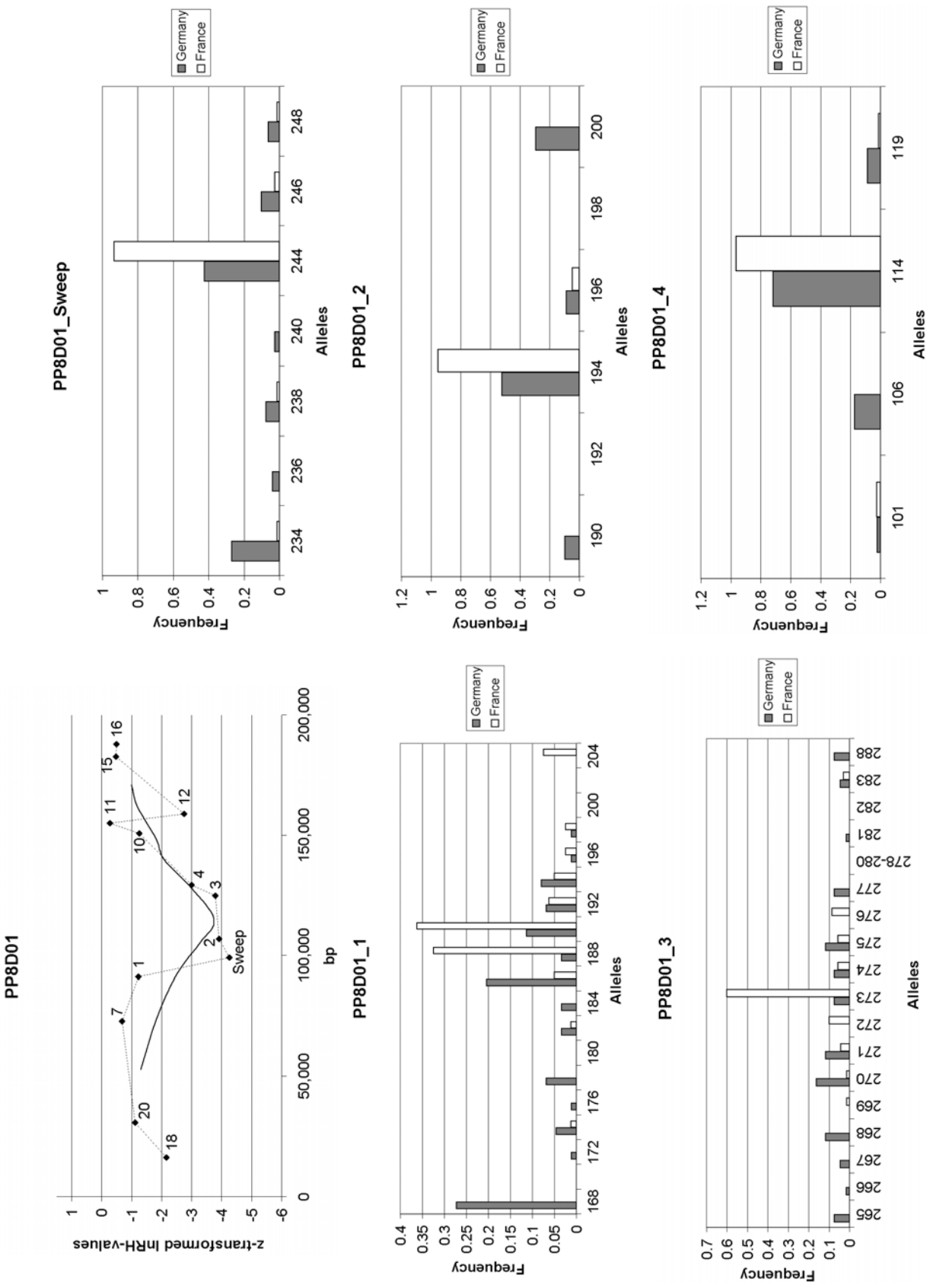


Chr. 7 / pp. 89,410 kb / r = 0.45



Chr. 11 / 38,981 kb / r = 0.66

Figure 4.6 Microsatellite loci genotyped for the two focal populations along 'Neutral regions'. The labeling of the Neutral Regions is from 1 to 9, Neutral Region 7 amplified in too few microsatellite loci and is therefore excluded.


PP8D01
z-transformed lnRH-values
bp
Sweep
PP8D01_Sweep
PP8D01_1
PP8D01_2
PP8D01_3
PP8D01_4
Germany
France
Frequency
Alleles

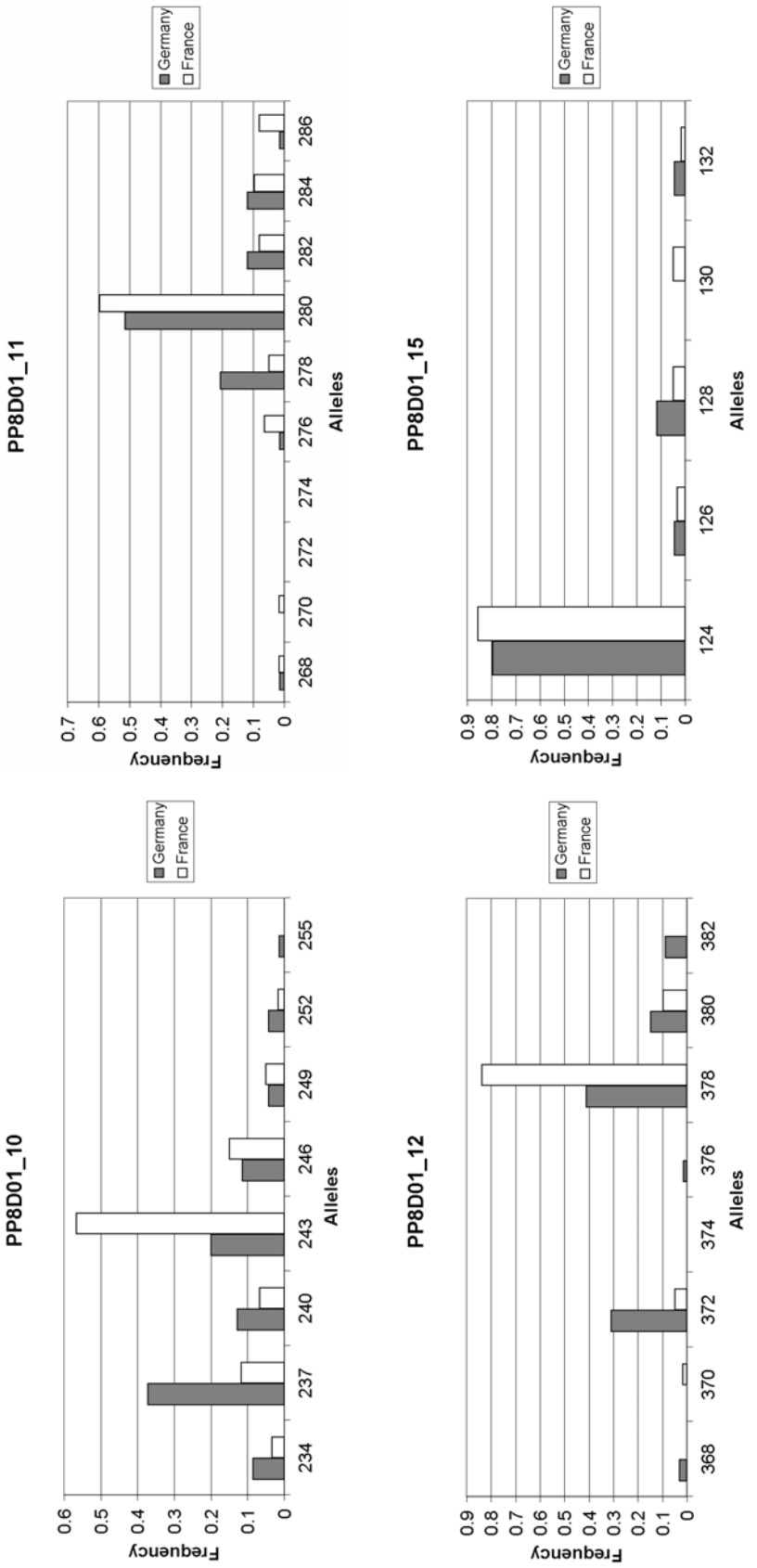
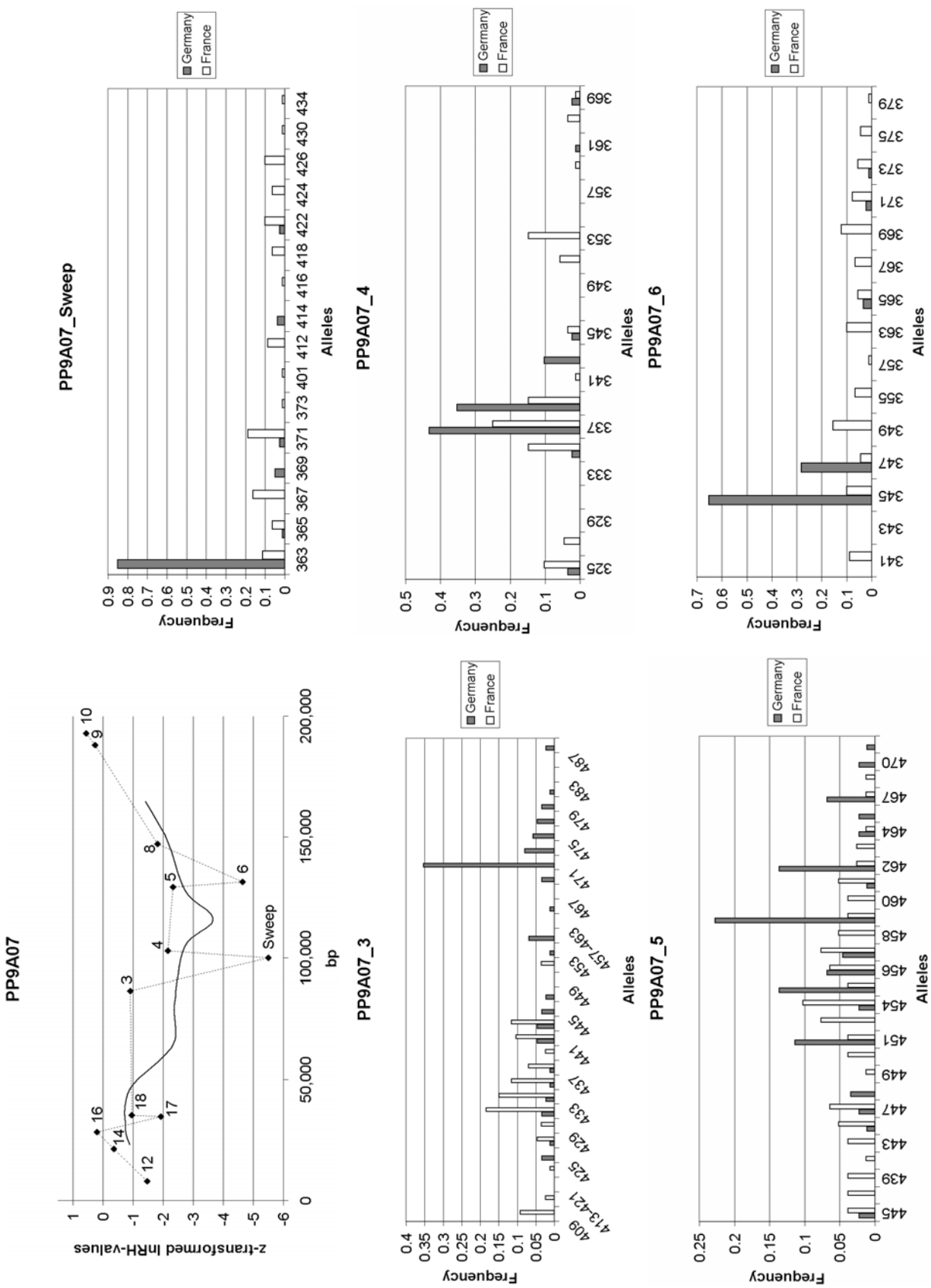


Figure 4.7 Allele frequency distribution for loci in two of the candidate regions

A Example 1 Locus PP8D01: The region shown below shows an extended sweep valley. The originally identified locus is labeled as "sweep" - the flanking loci are labeled with numbers. The allele distributions for each of these is shown below. Loci 10 an 11 appear to have recovered their variability after the sweep. Within the valley microsatellites with few alleles as well as microsatellites with numberous alleles show an extreme reduction in variability in the French population.


PP9A07
z-transformed lnRH-values
bp
Sweep
PP9A07_Sweep
PP9A07_4
PP9A07_6
PP9A07_3
PP9A07_5
Germany
France
Frequency
Alleles

B Example 2 Locus PP9A01: The region shows an extended sweep valley. The originally identified locus is labelled as "sweep" - the flanking loci are labeled with numbers. The allele distributions for each of these is shown below. Loci 4 and 5 appear to have recovered their variability after the sweep. The two major alleles in locus 4 are only one mutational step away, suggesting that one of them could have arisen by de novo mutation after the sweep. Locus 5 shows a large number of alleles, which is indicative of a generally high mutation rate, which could have led to a recovery of the variability after the sweep (note that the apparent new alleles are still grouped around the major allele, although not in the typical step-wise pattern).

than 160 kb. These numbers fit to earlier studies in *Drosophila*, where valleys of up to 100 kb were detected (Harr et al. 2002, Kauer et al. 2003, Beisswanger et al 2005).

4.4.4 Verification of sweep valleys

A comparison of variances in lnRH values (Schöfl and Schlötterer 2004) gave a significant difference between candidate and neutral regions (t-test p=0.0006, Figure 4.8). This analysis of variances represents an independent comparison. It is not influenced by differences in mutation rates because this parameter is eliminated in the lnRH statistic. Thus, the results validate the hypothesis that the contrasting pattern of candidate regions versus neutral regions is most probably not shaped by neutrality.

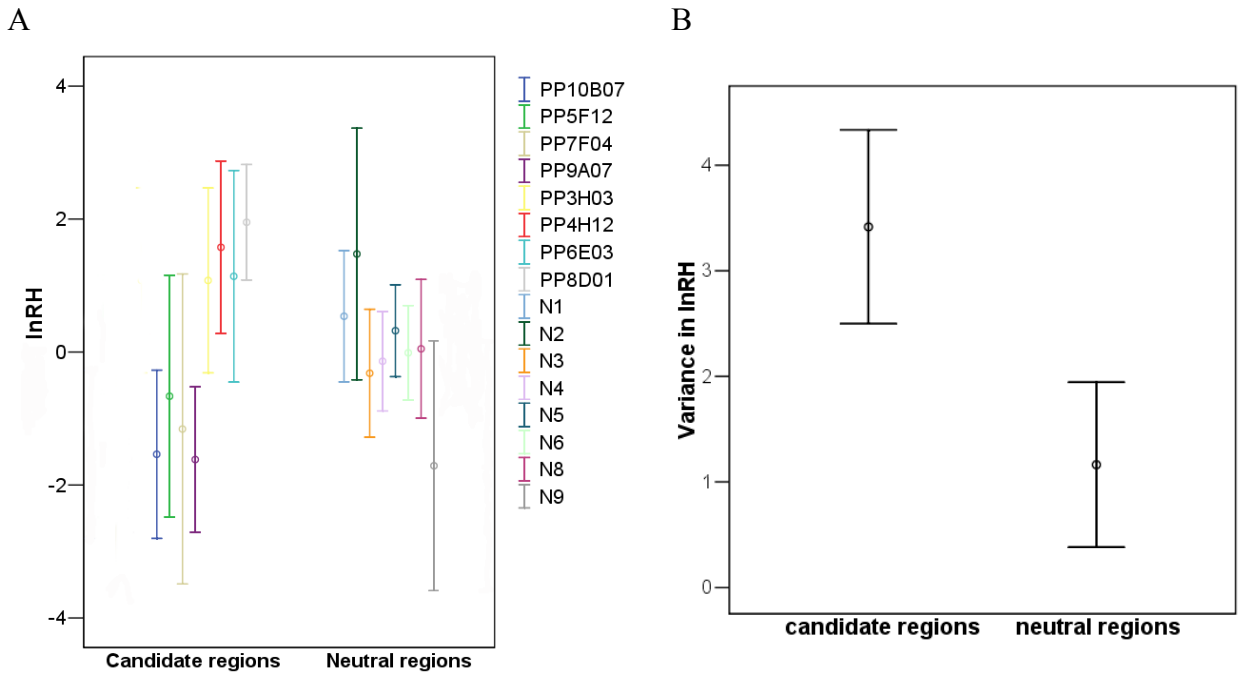


Figure 4.8 Comparison of variance in lnRH (error bars indicate the 95% confidence interval). (A) Distribution of lnRH values within the different regions of the candidate and the neutral areas. (B) Range of variance in lnRH values over the investigated candidate and neutral regions.

Table 4.3 Global Fst-values over the candidate regions and the eight neutral regions. The values significantly differ from eachother (t-test p=0.005; Kolmogorov-Smirnov p= 0.9).

Region	Size of the region [Kb]	Number of microsatellites in the region	global F_{st}
PP3H03	78	8	0.072957
PP4H12	77	9	0.176084
PP5F12	57	7	0.091722
PP6E03	117	10	0.109144
PP7F04	100	6	0.200342
PP8D01	172	13	0.120216
PP9A07	185	13	0.106477
PP10B07	180	10	0.13669
N1	100	8	0,061611
N2	100	3	0,096599
N3	100	6	0,064182
N4	120	9	0,050171
N5	100	4	0,060804
N6	80	5	0,035089
N8	180	6	0,064457
N9	150	6	0,116193

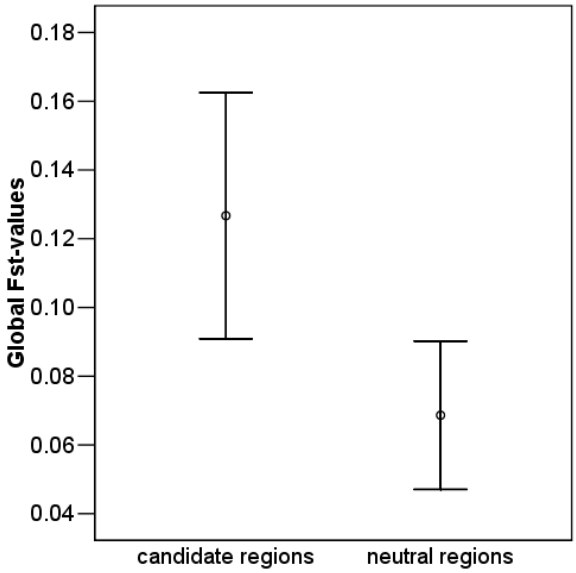


Figure 4.9 Comparison global F_{st}-values over candidate versus neutral regions (error bars indicate the 95% confidence interval). T-test confirmed a significant deviation of the two compared regional sets (p-value = 0.005).

Furthermore, we estimated global F_{ST} values (Weir and Cockerham 1984) over the candidate as well as the eight neutral regions using the software MSA (Dieringer and Schlötterer 2003). We tested whether F-statistic supports the deviation of candidate loci from the neutral expectation of differentiation. The t-test gave a significant result (Figure 4.9), a further indication for deviation from neutrality.

4.4.5 Bottleneck scenario

We included an independent dataset of 118 microsatellite loci distributed thoughout the genome (collected in Ihle et al. 2006) taken from the database of the Whitehead Institute/MIT Center for Genome Research and estimated the variance in heterozygosity for the German, the French, and the Kazakhstan population. The two *M. m. domesticus* populations are the 'potential bottleneck' populations, their variances in heterozygosity are 0.02061 (France) and 0.01656 (Germany), whereas an older, ancestral and therefore assumed to be 'non-bottlenecked' Kazakhstan population shows a higher value (0.02471), though this difference is not significant. Anyways, the ancestral population shows a higher variance than the candidate populations, an indication for a low probability of artificial valley formations due to a bottleneck. Consistent with the results of Schöfl and Schlötterer (2004) we do not detect a difference in mean heterozygosity between the ancestral (Kazakhstan 0.7859±0.1572) and the two derived popuations (Germany 0.7956±0.1287; France 0.7735±0.1436), which would be expected after a recent bottleneck event.

Table 4.4 Average Heterozygosity estimates from randomly picked loci (SD = standard deviation) (Ihle et al. 2006)

Parameter	Germany	France	Kazakhstan
N	118	118	118
Mean Heterozygosity	0.7956	0.7735	0.7859
SD Heterozygosity	0.1287	0.1436	0.1572
Variance	0.02061	0.01656	0.02471

Demographic events like bottlenecks have genome wide consequences, whereas selection is expected to have locus specific effects (Stajich and Hahn 2005). Thus, a deviation of candidate regions from the rest of the genome would validates the outstanding structure of these regions and clearly indicate that these regions have undergone a locus specific evolution. Additionally, comparisons of the variance in lnRH values over loci genotyped within the candidate regions with (1) the variance in lnRH over all loci genotyped in the eight neutral regions (for the comparison with the German candidates Levene test p=0.0325, for the comparison with the French candidates Levene test p=0.0331), (2) the variance in lnRH over 118 randomly

selected loci (for the comparison with the German candidates Levene test $p=2.91 \cdot 10^{-5}$, for the comparison with the French candidates Levene test $p=2.18 \cdot 10^{-5}$), and (3) the variance in lnRH over the loci in neutral regions together with the 118 randomly chosen loci (for the comparison with the German candidates Levene test $p=8.29 \cdot 10^{-5}$, for the comparison with the French candidates Levene test $p=9.13 \cdot 10^{-5}$) revealed significant results. Whereas the variances in lnRH values are homogenious between the candidate regions of the two investigated populations (Levene test p=0.823). This confirms the significant deviation of the candidate regions from the rest of the genome - a result that indicates locus specific evolution.

4.4.6 Selection coefficients

Based on the measured heterozygosity values of the flanking microsatellites within a valley and the local recombination rate, we estimated the selection coefficients. Values vary between 0.0007 and 0.0210. These numbers are averages over the locus wise estimation of two to twelve flanking microsatellites (Supplement A and B). For loci that have lnRH values larger than zero, the selection coefficient cannot be estimated. Thus, all loci that are positioned between the preselected candidate and the first locus exhibiting an lnRH value larger than zero are included in the regional selection coefficients.

Region	average selection coefficient
PP8D01	0.0210
PP4H12	0.0007
PP3H03	0.0008
PP6E03	0.0013
PP9A07	0.0018
PP10B07	0.0015
PP7F04	0.0029
PP5F12	0.0011

Table 4.5 Selection coefficients estimated for the eight candidate regions. The calculation is based on the heterozygosity values of flanking microsatellites and the recombination distance to the most reduced locus.

4.5 Discussion

Given the time frame within which the investigated populations were established, we can make a straightforward estimate of the frequency of positive selection events in natural populations of the house mouse. If we assume that the average window size of a sweep is not larger than 50kb, one should expect a total of 40,000 possible selectable loci (assuming a genome size of $2 \cdot 10^9$bp). From fossil records it is known that the house mouse invaded Western Europe no longer than 3,000 years ago (Cucchi et al. 2004). With an assumption of three generations per year (Karn et al. 2002), our lineages are separated by a total of 18,000 generations. With 0.42 - 0.83% sweep loci identified (four to eight out of 960), we arrive at an estimate of 0.93 - 1.85 selective sweeps per 100 generations, i.e. higher than the commonly stated maximum of 1 in 250 that was initially proposed by Haldane (1957).

Doubts about the authenticity of the identified sweep patterns because of possible bottleneck or drift events can be rejected. The lnRH statistic is a priori relatively refractory to the effects of bottlenecks (Kauer et al. 2003, Schlötterer 2002b, Schöfl and Schlötterer 2004). However, the consequences of bottlenecks comparable to that in *Drosophila* with the emigration out of Africa have yet to be sufficiently studied (Wiehe and Schlötterer, pers. comm.). Such Bottlenecks lead to a decrease in average heterozygosity as well as an initial increase in the variances of heterozygosities. Both factors would potentially inflate the number of signatures of selective sweeps in our statistics, although only under limited conditions (Wiehe and Schlötterer. pers. comm.). When we compare our populations with a more ancestral population from Kazakhstan, we detect no significant differences in the averages and variances of heterozygosities for a sample of 118 randomly picked microsatellite loci distributed throughout the genome (Table 4.4). This would suggest that there was no major bottleneck involved in the colonization of Middle Europe, which is in line with the proposal of Cucchi et al. (2005) that colonization became possible only after regular trading had started across the Mediterranean. Most importantly, however, the two populations that we compare in the lnRH statistic have very similar population parameters, i.e. we do not compare an ancestral and a derived population, but two that have recently split from each other. Thus, even if loci became randomly fixed during the colonization process, they would not show up in our analysis, because they would

be present in both populations and would therefore be cancled out in the lnRH statistic. Furthermore, if a subsequent bottleneck after the colonization of Middle Europe would have affected one of the populations, this would be visible in the average heterozygosity estimate, since the time was too short for many new mutations to occur, which could have reconstituted genome wide variability. Accordingly, we can exclude that demographic or historical population parameters influence our conclusions.

The shape of the sweep signature window is an additional important indicator for whether the signature reflects a true selective sweep. If local selection was the reason for a reduction in variability, one would expect that a closely flanking locus should also show some reduced variability (Maynard Smith and Haigh 1974, Kaplan et al. 1989, Kim and Stephan 2003). Taking this reasoning, we infer that at least the four loci where flanking variability was also lost (Figure 4.5) reflect true selective sweeps. On the other hand, we would expect for older sweeps that the signature is blurred and detected candidate loci are more isolated from flanking regions, i.e. the above criterion would be less likely to apply.

Fixation of alleles by neutral drift can be excluded as an explanation for variability reduction because of two major parameters: the age of the populations and the effective population size in mice. New alleles go to fixation by random drift after $4N_e$ generations. Therefore, with a given N_e of about 10,000, it would take about 40,000 generations, i.e. 13,000 years to fix new alleles. Fixation of existing alleles with a starting frequency of e.g. 0.5 roughly takes 12,000 generations, 4,000 years (Kimura and Ohta 1969). The age of the two investigated populations is not more than 9,000 generations (Karn et al. 2002, Cucchi et al. 2005), probably much younger, thus drift as a reason for the detected sweep candidates is fairly improbable.

lnRH values fluctuate within the candidate regions. These fluctuations seem to be due to differences in the mutation rates of the different investigated microsatellite loci. The mutation rates in microsatellites of humans and mice vary between 10^{-5} - 10^{-2} (see Schug et al. 1998). In the candidate region PP9A07 (Figure 4.5) loci PP9A07_4 and PP9A07_5, show much more variability than the two outer most extreme reduced loci (PP9A07_Sweep and PP9A07_6). By analysing the allele frequencies of these loci, we can conclude that the two inner microsatellites have a higher mutation rate than the two outer ones, and therefore recovered variability

within a shorter time (see Results 4.4.3). The extreme reduction in variability is therefore still present in the two loci with a lower mutation rate, whereas the two high mutating microsatellites already accumulated new alleles after the sweep event. Especially locus PP9A07_4 clearly depicts an accumulation of new alleles by stepwise mutation from the most frequent one. Applying different statistical tests (Variance in lnRH and F-statistic), we were able to show that the candidate regions significantly differ from neutrally evolving areas of about the same size. Thus, we reject neutrality as an explanation for the detected signatures.

Importantly, we have good reason to believe that we have in fact underestimated the true rate. Specifically, we want to point out that this number states a minimum of adaptive events for the following reasons: (1) with stringent statistics, we would detect only the most extreme values in a given window; we would not have detected most "soft sweeps" [i.e. positive selection events on alleles from the standing variation that were linked to more than one major allele of a flanking microsatellite (Hermisson and Pennings 2005)], and we would have missed relatively recent selection events because they would not have had time to build up a significant sweep signature, at least under the assumption of generally low selection coefficients; (2) the estimated number of generations per year (some authors postulate two generations per year in mice), and (3) the assumed divergence time (maximum of 3,000 years, but probably less).

Taken together, these factors lead to an underestimate of the true rate and are likely to change the above result, possibly by up to an order of magnitude. Previous estimates of the frequency of selection have been made by comparison of genome data in *Drosophila* (Smith and Eyre-Walker 2002, Andolfatto 2005). While the estimated rate of amino acid substitution driven by natural selection is about 1 in 450 generations from a comparison of *D. simulans* and *D. yakuba* (Smith and Eyre Walker 2002), or even one amino acid substitution every 800 ± 350 generations (Birne and Eyre-Walker 2004), the estimate that includes positive selection on non-coding DNA is up to 10 in 100 generations (Andolfatto 2005). Bazykin et al. (2004) have studied genome data from mouse and rat in this respect and also suggest that positive selection must be higher than previously anticipated. In addition to the adaptive events that go along with amino acid substitutions, we are able to detect adaptive changes that result from *cis* regulatory modifications. Such adaptive events cannot be

identified in comparative sequence analysis of old, diverged species. The footprints of reduced variability which go along with both types of evolutionary changes are still present in the young investigated populations, and can be identified in our genome screen for signatures of selective sweeps. Therefore we expect our result to reveal a higher frequency. Thus, although we compare different populations rather than different species and use a very different approach, we arrive at similar conclusions. In fact, it has been shown by Maynard-Smith (1968) that selection frequencies of 1 in 10 generations may be feasible, if one takes epistasis effects into account, although this issue is still not resolved (Nei 2005). It has been noted that such a high rate of positive selection in natural populations would have consequences for the estimation of population genetical parameters under the neutral model (Maynard Smith and Haigh 1974, Gillespie 2000).

By analysing the pool pattern of the other than the two Western European populations, we are able to detect about the same number of sweep candidates in an old ancestral population, Kazakhstan. This leads us to the conclusion that positive selection events do not necessarily occur exclusively with strong changes in the environment, e.g. with invasions into new habitats, but that selective sweeps seem to be a continuous background process that can be observed in all populations. By comparing the pool patterns of the two *M. m. musculus* populations (from Kazakhstan and the Czech Republic), we identified two unambiguous candidates for Kazakhstan specific signatures of selective sweeps. Thus, positive selection events are not exclusively present in populations that have recently undergone environmental changes, but can also be observed in old ancestral ones. Similar to these results, Schlötterer (2002b) found the same number of significant signatures of selective sweeps in the African and the non-African human population, which is in contrast to the model of out-of-Africa-associated adaptive mutations, but indicating that also in humans selection is not exclusively connected to environmental changes. Similar results were found in a recently published paper about a SNP based analysis in humans, were strong selection was detected in a Sub-Saharan African population (Voight et al. 2006). In contrast to this, Kayser et al. (2003) investigated 332 microsatellite loci and identified ten selective sweep loci in a European population but only one in an African population. In the recently published paper by Haddrill et al. (2005) the authors postulate that their results imply that highly reduced variation

observed at some loci in genome wide scans, e.g. Harr et al 2002, Glinka et al 2003, Kauer et al 2003, might be more easily explained by a bottleneck in the history of European populations than by recurrent selective sweeps. Thus, the excess of sweep signatures in derived populations in comparison to ancient ones might be an artefact. We therefore postulate that adaptive events can be observed in all populations more or less equally and that adaptation is not restricted to changes in the environment, it is a continuous background process.

Based on the size of the identified candidate regions and the age of the populations, we can conclude that the selective pressure acting on these loci is small. None of the windows is very broad, implying that the selection coefficients are roughly in balance with the recombination rates, effectively isolating positive selection on one locus from neighbouring loci. The calibration scale for values of selection coefficient 's' is the effective population size. In small populations selection has to be stronger to act efficiently due to the larger impact of drift (Maynard Smith and Haigh 1974). Positive selection is acting if $4N_e s > 1$. Given N_e equals 10,000, a minimal s of $2.5 \cdot 10^{-5}$ is required for the presence of positive selection. This number describes the lowest possible selection coefficient that can act efficiently in a house mouse population. The minimal required selection coefficient to fix an allele within the known maximal divergence time of 9,000 generations is $2.2 \cdot 10^{-3}$, estimated from $t=(2/s) \cdot \ln(2N_e)$ (Stephan et al. 1992). Using a formula proposed by Stephan et al. (1992) one can estimate selection coefficients for the respective sweep valleys based on the measures of heterozygosity and local recombination rate (see Methods). The results for our candidate regions range from 0.0007 to 0.021 (Table 4.5). Given the absence of large chromosomal regions showing reduced variability and the order of magnitude of our calculated s-values, we hypothesize that adaptive events are generally associated with small selection coefficients. There is no evidence for very strong selection. This confirms long standing speculations that strong selection on single loci is likely to be rare because of pleiotropic effects and possibility of linkage to maladaptive alleles in flanking loci (Barton and Partridge 2000).

Artificial selection experiments show invariably that any polymorphic population harbours enough standing variation to allow the selection of traits in almost every conceivable direction (Barton and Partridge 2000). If most of the selection in natural populations would also be derived from the standing variation

rather than new mutations, we would expect that signatures of selective sweeps are blurred because a beneficial mutation could be linked to more than one microsatellite allele. In fact, we find that none of the eight preselected candidate loci that we identified shows a single fixed allele. There are always one or more low frequency alleles present (see Figure 4.4). These alleles are usually distant from the major allele suggesting that they have not arisen via new mutations after fixation of one allele, since new mutations at microsatellite loci are expected to occur predominantly in a step-wise fashion, i.e. new alleles would flank the major one.

There is the potential that such alleles appear due to recombination events during the fixation phase, although this is unlikely because of their low frequency. Recombination takes place in the early phase of a selective sweep. Thus, alleles that become linked to the beneficial variant in this phase should be at a higher frequency at the end of a selective sweep. Additionally, since we detect the distal alleles consistently in all eight significant outliers, recombination becomes unlikely because this would mean all detected loci would have undergone similar recombination events leading to low frequency of additional alleles. It is much more likely that there is a general mechanism behind these consistent results. Thus, our data are compatible with the assumption that beneficial alleles come from the standing variation, although we can not exclude new mutations either.

We postulate that positive selection events occur with a minimal frequency of one sweep every 100 generations, and are generally associated with small selection coefficients. Strong selection is rare, which is in line with previously assumed consequences of pleiotropic effects and an accumulation of negative mutations that might hitchhike on large chromosomal regions which are fixed during a sweep. Furthermore, we conclude that beneficial variants are generally taken from the standing variation and that positive selection events are not exclusively linked to changes in the environment, but represent a continously acting background process in all populations. Populations are never perfectly adapted, adaptation is always on its way to optimization.

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5 Supplement

- Supplement 1 Calculation of selection coefficiens
- Supplement 2 Primer sequences of the 960 screened microsatellite loci.
- Supplement 3 Primer sequences of the microsatellite loci genotyped along the candidate regions.
- Supplement 4 Primer sequences of the loci genotyped along the 'neutral regions'.
- Supplement 5 Allele frequencies of the loci genotyped in candidate region PP10B07 (German sweep).
- Supplement 6 Allele frequencies of the loci genotyped in candidate region PP7F04 (German sweep).
- Supplement 7 Allele frequencies of the loci genotyped in candidate region PP5F12 (German sweep).
- Supplement 8 Allele frequencies of the loci genotyped in candidate region PP4H12 (French sweep).
- Supplement 9 Allele frequencies of the loci genotyped in candidate region PP3H03 (French sweep).
- Supplement 10 Allele frequencies of the loci genotyped in candidate region PP6E03 (French sweep).

Supplement 1 Calculation of selection coefficiens

A For the regions that show a signature of a selective sweep in the German population.

Locus	Het.Ger	Het.Fra	lnRH	Distance to sweep locus [bp]	RR [5 Mb]	recombination distance [M]	HGer/HFra	Selection coefficient per locus	average selection coefficient
PP9A07_12	0.7147	0.8367	-1.4700	-92277	0.4400	0.0004	0.8541		
PP9A07_14	0.6248	0.6594	-0.3604	-78814	0.4400	0.0003	0.9475		
PP9A07_16	0.7959	0.7684	0.2071	-71873	0.4400	0.0003	1.0358		
PP9A07_17	0.8005	0.9073	-1.9265	-65489	0.4400	0.0003	0.8822	0.0027	
PP9A07_18	0.9056	0.9343	-0.9513	-65029	0.4400	0.0003	0.9693	0.0016	
PP9A07_3	0.8571	0.8982	-0.9028	-13653	0.4400	0.0001	0.9543	0.0004	
PP9A07_S	0.2756	0.8968	-5.4968	0	0.4400	0.0000	0.3073		
PP9A07_4	0.6839	0.8634	-2.1569	2799	0.4400	0.0000	0.7921	0.0002	
PP9A07_5	0.8924	0.9584	-2.3268	29056	0.4400	0.0001	0.9311	0.0009	
PP9A07_6	0.4986	0.9179	-4.6460	31187	0.4400	0.0001	0.5432	0.0035	
PP9A07_8	0.5772	0.7815	-1.8129	47037	0.4400	0.0002	0.7385	0.0031	
PP9A07_9	0.8559	0.8316	0.2697	87731	0.4400	0.0004	1.0292		
PP9A07_10	0.4495	0.3411	0.5599	92601	0.4400	0.0004	1.3180		0.0018
PP10B07_14	0.8745	0.8542	0.2540	-95949	0.3424	0.0003	1.0238		
PP10B07_15	0.3474	0.6864	-2.3355	-76769	0.3424	0.0003	0.5061		
PP10B07_17	0.8948	0.8390	0.9080	-59764	0.3424	0.0002	1.0666		
PP10B07_18	0.7470	0.8565	-1.4756	-51090	0.3424	0.0002	0.8722	0.0017	
PP10B07_4	0.4444	0.7134	-1.9749	-41997	0.3424	0.0001	0.6228	0.0029	
PP10B07_6	0.6173	0.7666	-1.3730	-35067	0.3424	0.0001	0.8053	0.0015	
PP10B07_S	0.3718	0.9244	-5.6120	0	0.3424	0.0000	0.4023		
PP10B07_3	0.5935	0.6842	-0.7778	7943	0.3424	0.0000	0.8675	0.0003	
PP10B07_2	0.8399	0.9334	-2.1706	35086	0.3424	0.0001	0.8998	0.0010	
PP10B07_9	0.8527	0.8910	-0.8146	84872	0.3424	0.0003	0.9570	0.0018	0.0015

Locus	Het.Ger	Het.Fra	lnRH	Distance to sweep locus [bp]	RR [5 Mb]	recombination distance [M]	HGer/HFra	Selection coefficient per locus	average selection coefficient
PP7F04_5	0.6940	0.6107	0.5357	-77064	0.8094	0.0006	1.1363		
PP7F04_7	0.7867	0.8390	-0.7801	-66322	0.8094	0.0005	0.9377	0.0038	
PP7F04_2	0.8197	0.8699	-0.8803	-35473	0.8094	0.0003	0.9423	0.0020	
PP7F04_S	0.3628	0.9084	-5.2187	0	0.8094	0.0000	0.3994		
PP7F04_3	0.8741	0.7965	1.0472	13444	0.8094	0.0001	1.0974		
PP7F04_4	0.5366	0.7399	-1.6474	22567	0.8094	0.0002	0.7252		0.0029
PP5F12_3	0.8571	0.7661	1.0880	-16790	1.0220	0.0002	1.1188		
PP5F12_4neu	0.5394	0.7871	-2.1240	-11869	1.0220	0.0001	0.6853	0.0021	
PP5F12_8	0.8707	0.9048	-0.8235	-3133	1.0220	0.0000	0.9623	0.0002	
PP5F12_S	0.4545	0.8949	-4.3378	0	1.0220	0.0000	0.5079		
PP5F12_5	0.9159	0.8706	0.9135	12688	1.0220	0.0001	1.0520		
PP5F12_6	0.8673	0.8320	0.4607	23867	1.0220	0.0002	1.0425		
PP5F12_7	0.9020	0.8899	0.1738	40554	1.0220	0.0004	1.0137		0.0011

B For the regions which show a signature of a selective sweep in the French population.

Locus	Het.Ger	Het.Fra	lnRH	Distance to sweep locus [bp]	RR [5 Mb]	recombination distance [M]	HFra/HGer	Selection coefficient per locus	average selection coefficient
PP8D01_18	0.8327	0.5910	-2.1614	-83063	2.0560	0.0017	0.7097	0.0274	
PP8D01_20	0.8588	0.7661	-1.1160	-68537	2.0560	0.0014	0.8921	0.0125	
PP8D01_7	0.6564	0.5450	-0.6763	-26312	2.0560	0.0005	0.8303	0.0060	
PP8D01_1	0.8589	0.7563	-1.2195	-7993	2.0560	0.0002	0.8805	0.0015	
PP8D01_S	0.7353	0.1277	-4.2583	0	2.0560	0.0000	0.1737		
PP8D01_2	0.6314	0.0878	-3.9182	7725	2.0560	0.0002	0.1390	0.0210	
PP8D01_3	0.9188	0.6032	-3.7878	25623	2.0560	0.0005	0.6564	0.0098	
PP8D01_4	0.4447	0.0703	-2.9970	30023	2.0560	0.0006	0.1580	0.0711	
PP8D01_10	0.7925	0.6452	-1.2539	51519	2.0560	0.0011	0.8141	0.0125	
PP8D01_11	0.6743	0.6245	-0.2755	55816	2.0560	0.0011	0.9262	0.0087	
PP8D01_12	0.7151	0.2893	-2.7489	59640	2.0560	0.0012	0.4045	0.0469	
PP8D01_15	0.3569	0.2676	-0.4749	83325	2.0560	0.0017	0.7498	0.0245	
PP8D01_16	0.9139	0.8893	-0.4893	88546	2.0560	0.0018	0.9731	0.0100	0.0210
PP4H12_8	0.5720	0.6068	0.3399	-44850	0.2200	0.0001	1.0609		
PP4H12_1	0.9321	0.7291	-3.2046	-43261	0.2200	0.0001	0.7822	0.0012	
PP4H12_10	0.8116	0.6277	-1.6168	-42515	0.2200	0.0001	0.7734	0.0012	
PP4H12_3	0.6371	0.4532	-1.1026	-37606	0.2200	0.0001	0.7114	0.0013	
PP4H12_12	0.8634	0.7604	-1.2529	-13468	0.2200	0.0000	0.8808	0.0003	
PP4H12_2	0.8973	0.7150	-2.3633	-6031	0.2200	0.0000	0.7968	0.0002	
PP4H12_S	0.5389	0.0250	-4.8689	0	0.2200	0.0000	0.0464		
PP4H12_5	0.5158	0.4747	-0.1526	10427	0.2200	0.0000	0.9204	0.0002	
PP4H12_4	0.7957	0.7908	0.0435	21814	0.2200	0.0000	0.9938	0.0002	0.0007

Locus	Het.Ger	Het.Fra	lnRH	Distance to sweep locus [bp]	RR [5 Mb]	recombination distance [M]	Hfra/HGer	Selection coefficient per locus	average selection coefficient
PP3H03_2	0.8454	0.8507	-0.1866	-37472	0.4400	0.0002	1.0064		
PP3H03_3	0.8864	0.7886	1.3824	-31256	0.4400	0.0001	0.8897	0.0012	
PP3H03_S	0.7092	0.1187	4.1250	0	0.4400	0.0000	0.1673		
D10Mit23	0.7427	0.5547	1.3532	5638	0.4400	0.0000	0.7469	0.0004	
PP3H03_5	0.8304	0.8315	-0.1178	25420	0.4400	0.0001	1.0013		
PP3H03_6	0.8804	0.8156	0.9294	36108	0.4400	0.0002	0.9264		
PP3H03_7	0.8055	0.7926	0.0547	40928	0.4400	0.0002	0.9839		0.0008
D1Mit305	0.8359	0.9030	1.3470	-24296	0.5094	0.0001	1.0802		
PP6E03_1	0.6688	0.7155	0.4930	-14634	0.5094	0.0001	1.0698		
PP6E03_3neu	0.7840	0.6643	-1.0091	-5921	0.5094	0.0000	0.8473	0.0003	
PP6E03_S	0.7247	0.0737	-4.9066	0	0.5094	0.0000	0.1017		
PP6E03_2	0.6251	0.5726	-0.2620	20642	0.5094	0.0001	0.9160	0.0008	
D1Mit440	0.7774	0.6487	-1.0559	32840	0.5094	0.0002	0.8344	0.0018	
PP6E03_5	0.8139	0.7620	-0.4974	58432	0.5094	0.0003	0.9363	0.0021	
PP6E03_6	0.7429	0.8153	0.9115	65638	0.5094	0.0003	1.0975		
PP6E03_7	0.7855	0.0977	-5.1503	67291	0.5094	0.0003	0.1244		
PP6E03_9	0.9317	0.8778	-1.2640	92255	0.5094	0.0005	0.9422		0.0013

Supplement 2 Primer sequences of the 960 screened microsatellite loci.

Label		Marker	F-Primer	Sequence	R-Primer	Sequence
PP1	A01	D6Mit115	D6Mit115F	ccatttaataagtgatccctctgg	D6Mit115R	tgtcacaccacaatgggc
PP1	B01	D6Mit364	D6Mit364F	tagaccttgtctcaaatgtatgtgtg	D6Mit364R	cccctgatgctgtaggtgtt
PP1	C01	D6Mit138	D6Mit138F	gctcttattaatgaagaagaaggagg	D6Mit138R	caaagaaagcatttcaagactgc
PP1	D01	D6Mit139	D6Mit139F	atagaaggcgagaactaacccc	D6Mit139R	tgtttctgcccctgtagttg
PP1	E01	D6Mit365	D6Mit365F	gtctggtgtatttgcatatatggg	D6Mit365R	gcaggcagacacacagacaa
PP1	F01	D6Mit391	D6Mit391F	ttctctcagtcttgtctgtgtaca	D6Mit391R	gtgaggctcaaagaaagggc
PP1	G01	D6Mit73	D6Mit73F	atacactttgacacaagagcaagg	D6Mit73R	agcacagaggtcagaaactgc
PP1	H01	D6Mit326	D6Mit326F	tgactggaggacagagattgg	D6Mit326R	atgtccatttaagtcttttctggg
PP1	A02	D6Mit38	D6Mit38F	cttagtgcgtgtaaggcaagg	D6Mit38R	gactgctgagctagtgccct
PP1	B02	D6Mit199	D6Mit199F	gcccttcctactcaaaataaatacc	D6Mit199R	gggtagttcaaattaaacctggg
PP1	C02	D6Mit175	D6Mit175F	gttagtgagatccaaagccacc	D6Mit175R	gccaccatctcaaccctg
PP1	D02	D6Mit210	D6Mit210F	ttagaggaagagaactgatagaatgtg	D6Mit210R	attaacttcaaggagaagcccc
PP1	E02	D6Mit315	D6Mit315F	agaaaatagagtcgatctagacacaca	D6Mit315R	atgaaagtcaggttgtcggg
PP1	F02	D6Mit333	D6Mit333F	tcctcactacaattcatctattactgc	D6Mit333R	tgcttctggtataggcagttagg
PP1	G02	D6Mit200	D6Mit200F	catcaggtgtcttcaggttctg	D6Mit200R	tcccctctatccttactgttgc
PP1	H02	D6Mit288	D6Mit288F	agcactggctagagaatcatcc	D6Mit288R	cattcgactcttcaggccat
PP1	A03	D6Mit302	D6Mit302F	aatgaccctggttagtgtcagg	D6Mit302R	gaattccattcgaggggc
PP1	B03	D6Mit254	D6Mit254F	agtgtccctagggggtgg	D6Mit254R	ggggccttagaggtagcaac
PP1	C03	D6Mit44	D6Mit44F	cccgtgtccagggtactg	D6Mit44R	gcatggtaccccagcttcta
PP1	D03	D6Mit193	D6Mit193F	tagtacagaaagttattctggatgtgg	D6Mit193R	taagccacactgattgatgtcc
PP1	E03	D6Mit362	D6Mit362F	gaaaactgtcctctgacatttataagc	D6Mit362R	ttgatcttgagggtttaaaatcg
PP1	F03	D6Mit253	D6Mit253F	gtcaacatctatgttccactcagg	D6Mit253R	ctcatgcacgtctatacacaagc
PP1	G03	D6Mit308	D6Mit308F	ttactagagaacttgggagaaccg	D6Mit308R	ctactgtcgccacctaacctg
PP1	H03	D6Mit119	D6Mit119F	gggctagtttctcatgaagtaagc	D6Mit119R	tacattttatcactaggtgaatgtgtg
PP1	A04	D6Mit29	D6Mit29F	cttctttacacctgtatggcacc	D6Mit29R	ggttggtcactgcaggagtt
PP1	B04	D6Mit323	D6Mit323F	gactgtaaatggatccttcctcc	D6Mit323R	acctaacaacctacttcaagaaaagc
PP1	C04	D6Mit46	D6Mit46F	ttagaactgtgaagagggtcagc	D6Mit46R	tggctgtttgttaattcgacc
PP1	D04	D6Mit141	D6Mit141F	cacctctcaccaccaccac	D6Mit141R	acctgcatttcccacacc
PP1	E04	D6Mit153	D6Mit153F	attctgagtatatgagcctctggg	D6Mit153R	gatgttactaagtaagatacttcgccg
PP1	F04	D6Mit21	D6Mit21F	ctgggattaaagactaccatgagc	D6Mit21R	cacctgactctaatccctgtcc
PP1	G04	D3Mit215	D3Mit215F	taaacatctagaagatgctgcagg	D3Mit215R	ctgcatggccaggactagtt
PP1	H04	D3Mit240	D3Mit240F	ccaattgggataagatactgttcc	D3Mit240R	gtaacgtaaccttactgctgtgtagg
PP1	A05	D3Mit286	D3Mit286F	tcctactggaatccaatacaagtg	D3Mit286R	gtggtgtgtcttgacattctatatcc
PP1	B05	D6Mit5	D6Mit5F	cacggagaggacctacatgc	D6Mit5R	agctgctcgtctccacactt
PP1	C05	D6Mit55	D6Mit55F	caagcagagcacctagggtc	D6Mit55R	tcactgcaggattggtcttg
PP1	D05	D6Mit280	D6Mit280F	acagcaggtagactctgtacaatca	D6Mit280R	gcataaagtgaggtttttatttgtaca
PP1	E05	D6Mit300	D6Mit300F	tgagatcctacctcattacacttcc	D6Mit300R	ttggttcatagcctttcatgg
PP1	F05	D6Mit372	D6Mit372F	ttaatacacttaggtgtggctctcc	D6Mit372R	gagaggcatatagaaaaggataatgc
PP1	G05	D3Mit160	D3Mit160F	caatgacacagcatgtctaacttg	D3Mit160R	ttaagaactactcaatgctgcacc
PP1	H05	D3Mit99	D3Mit99F	ggagactaaaatgcaagactgatg	D3Mit99R	cctcacatgtaattagcacatgc
PP1	A06	D3Mit293	D3Mit293F	acctgcacgtattttatgtctactc	D3Mit293R	gagtgtgcatgtatattttgtttgg
PP1	B06	D3Mit307	D3Mit307F	gaacttgtagcagaaagaaagatgg	D3Mit307R	tggtattttaagaatcgctcataca
PP1	C06	D3Mit155	D3Mit155F	aagcaacattatacagactgaacagg	D3Mit155R	aagttcacagcctccccc
PP1	D06	D3Mit305	D3Mit305F	gaatctaacttggggacctgc	D3Mit305R	tacagaaaggtgattggagttgg
PP1	E06	D3Mit323	D3Mit323F	tatacacacactcgagcacgc	D3Mit323R	gctaaagtaacagagtgacctttgg
PP1	F06	D3Mit42	D3Mit42F	tgacctccagagagtcttcca	D3Mit42R	taagcagctgagactcaagtgg
PP1	G06	D3Mit251	D3Mit251F	atccatacacacagagacatacaca	D3Mit251R	aacagggactagtgtggagtaagc

PP1	H06	D3Mit279	D3Mit279F	ttacccctctctttctttaagtgtg	D3Mit279R	atccctagggcttcttgctc
PP1	A07	D3Mit283	D3Mit283F	ctcagcattgtgttttgtatacagc	D3Mit283R	cattcattttcatcaaagtatatttcc
PP1	B07	D3Mit194	D3Mit194F	gtttttgtgatgttacatactggacc	D3Mit194R	acaactcatccccctcctct
PP1	C07	D3Mit338	D3Mit338F	gtccaataccaagaagtcagcc	D3Mit338R	caatcctaatctgaaacagaattcc
PP1	D07	D3Mit353	D3Mit353F	tccccagtagtgagaaatcagg	D3Mit353R	tatgttcatgttcttccatttctagg
PP1	E07	D3Mit32	D3Mit32F	caccctggttaactcagaaagg	D3Mit32R	gcacttgtgtttcatgtcactg
PP1	F07	D3Mit226	D3Mit226F	gcatagaggacttctttgagaataca	D3Mit226R	tctgaactttgctgcccc
PP1	G07	D3Mit278	D3Mit278F	aactaccatctaaaacatcctctgtg	D3Mit278R	agatccctagagaaacagaactgg
PP1	H07	D3Mit298	D3Mit298F	ttaacttctggcttctatacacatgc	D3Mit298R	acaggaacatgcacacgtgt
PP1	A08	D3Mit12	D3Mit12F	tagaccaatcttgggagtgtcc	D3Mit12R	ggaaaagcataagaaacaaccg
PP1	B08	D3Mit257	D3Mit257F	cctagcgcaggaatagttaacc	D3Mit257R	acaaacagaacaaaacaaaaagtcc
PP1	C08	D3Mit28	D3Mit28F	gatgagagattctgatgtggagg	D3Mit28R	ccagcctcagtatctcaaaacc
PP1	D08	D3Mit29	D3Mit29F	gatgagagattctgatgtggagg	D3Mit29R	ccagcctcagtatctcaaaacc
PP1	E08	D3Mit220	D3Mit220F	caggacactcagtgcccc	D3Mit220R	tctcaagcatcatgtgttcatg
PP1	F08	D3Mit25	D3Mit25F	gtctgggtcgtcagtggc	D3Mit25R	tggaggctaccatctccaag
PP1	G08	D3Mit269	D3Mit269F	cagtggtcttatctatctatttcacca	D3Mit269R	taacatgtgtatgtatgagtgtgtgtg
PP1	H08	D3Mit303	D3Mit303F	aggtcctaggttcttattttctacaca	D3Mit303R	atgcctgctctgtgtgtgtc
PP1	A09	D3Mit313	D3Mit313F	gaaatagtacagagaaacagacacaca	D3Mit313R	cattctaaaacatactgtgcaggg
PP1	B09	D3Mit331	D3Mit331F	atatcactgagtacacacatgattgtg	D3Mit331R	gagtacacttgatgtcaaccaacc
PP1	C09	D3Mit28	D3Mit28F	gatgagagattctgatgtggagg	D3Mit28R	ccagcctcagtatctcaaaacc
PP1	D09	D3Mit297	D3Mit297F	gtgctagcaaggaggatcttatg	D3Mit297R	ggtaaagatgaggaatatcagtctca
PP1	E09	D3Mit211	D3Mit211F	catgtactgtgtaagttcatgctcc	D3Mit211R	ctctacatatatgctgtgtcatgtgc
PP1	F09	D3Mit263	D3Mit263F	aacaggtggatgttctagtcatagc	D3Mit263R	caaccagggtctccctgata
PP1	G09	D3Mit342	D3Mit342F	ccagggatctacataactaatgtgc	D3Mit342R	ctgagattcctcaagggcag
PP1	H09	D3Mit351	D3Mit351F	attcacacagtgacaggtacacg	D3Mit351R	gacaaaaaggtgaaaactcatgg
PP1	A10	D3Mit28	D3Mit28F	gatgagagattctgatgtggagg	D3Mit28R	ccagcctcagtatctcaaaacc
PP1	B10	D3Mit103	D3Mit103F	ccaggggtggtggtcttac	D3Mit103R	tgtcaggtgcccaggtct
PP1	C10	D3Mit302	D3Mit302F	ccccttctctgtcctgctc	D3Mit302R	cagaagtctatgcatagatctcaagg
PP1	D10	D3Mit322	D3Mit322F	catgctgtggagtgtacatacttg	D3Mit322R	ccaagatgacccccctaacaa
PP1	E10	D3Mit332	D3Mit332F	agcctaagttagttgttcctgtgg	D3Mit332R	tgtaaaaaccagaggagacaagg
PP1	F10	D3Mit147	D3Mit147F	tctgcctctgttagatagatatccg	D3Mit147R	ttgttcatctatcctctgaagttcc
PP1	G10	D7Mit342	D7Mit342F	cagtgagaccctaacttaaaaaactg	D7Mit342R	gcaagtgctcttaactactagcaca
PP1	H10	D7Mit267	D7Mit267F	ctctttctgttacatggttagatttcc	D7Mit267R	aaagacagttgaagttgacttctgg
PP1	A11	D7Mit309	D7Mit309F	tgataaggaccctacaagcacc	D7Mit309R	cacagagatggacagatacagaca
PP1	B11	D7Mit279	D7Mit279F	agcaacatctcaccctataccc	D7Mit279R	tagtcctaccaacagcctgtca
PP1	C11	D7Mit347	D7Mit347F	ctgagttccctaaagtctgtttcc	D7Mit347R	aacccagtgtccaccct
PP1	D11	D7Mit163	D7Mit163F	ggacagacaccctcaccg	D7Mit163R	cggctgtgagagcatagtga
PP1	E11	D7Mit319	D7Mit319F	gatctgaagacacatgatctgagg	D7Mit319R	gttaaacttcagaagtgtgagactgc
PP1	F11	D7Mit16	D7Mit16F	ctggtctctgtccttggagc	D7Mit16R	aaagaaaatattcttgttgccagc
PP1	G11	D7Mit124	D7Mit124F	gtcagacattggcttaggatcc	D7Mit124R	ggtttgtgcgctctctctct
PP1	H11	D7Mit358	D7Mit358F	ttagtgtgcttaaggtctacacacg	D7Mit358R	aacaaatactagagacaaacgtgtgc
PP1	A12	D7Mit44	D7Mit44F	ttctggcctctgtgaagtagtg	D7Mit44R	gtgaaaccatggtgcagatg
PP1	B12	D7Mit166	D7Mit166F	tatataaatctatcaagacacaccccc	D7Mit166R	agtatctgattttgatctctggcc
PP1	C12	D7Mit361	D7Mit361F	tactaccaatgctaagcgctacc	D7Mit361R	accagagtttgtgcccattc
PP1	D12	D7Mit362	D7Mit362F	ggggttagaccagttgaccc	D7Mit362R	tttgcatatgtgtgtatacatgtgg
PP1	E12	D8Mit353	D8Mit353F	tctggcttgtagtatctgtattcaca	D8Mit353R	atgaaggtgtgtgtgtatatatgtgc
PP1	F12	D8Mit35	D8Mit35F	gagtcctacaagttgtgccttg	D8Mit35R	ggagataaggagacaagtgtccc
PP1	G12	D8Mit125	D8Mit125F	atcgctctatctactcatctattcaca	D8Mit125R	gaccctgactcttaatcctagtgc
PP1	H12	D8Mit341	D8Mit341F	ctggctgcatacatacagatatataca	D8Mit341R	tgattgcttgtaggtatacaggtaca
PP2	A01	X_2	X_2F	actctcaggaatgtgttgcgtc	X_2R	tggagagaccttgggctaccca
PP2	B01	X_3	X_3F	cttcgccaagcgggtctgcagc	X_3R	tccggtctccagtgcctgggtc

PP2	C01	X_4	X_4F	actaaagctgccgatccgcctg	X_4R	caaagacagcccaggcaccttc
PP2	D01	X_5	X_5F	ctcgcatactcaaatgacccac	X_5R	ctaataagcccgagtaggccac
PP2	E01	X_6	X_6F	gaatggcctctctcaggaggtg	X_6R	tctccttggccctgtgcttcag
PP2	F01	X_7	X_7F	tgcagggatactggctcaaacc	X_7R	agttccaggacccactagcctg
PP2	G01	X_8	X_8F	tcacatcttgaaccactagcag	X_8R	tgaatacttggtcctgagttgc
PP2	H01	X_9	X_9F	ttggagactgtaatcccaaacc	X_9R	aactagctgggtggtcacagtc
PP2	A02	X_12	X_12F	agaggccagtaaagctggaagg	X_12R	tggctggcagtgaactcgttgc
PP2	B02	X_13	X_13F	tccttgggaagactgaaccacc	X_13R	agctagtctgaagcagccacac
PP2	C02	X_14	X_14F	agcaagacaggatagcacaacg	X_14R	atctcagcacctgtcccaagtg
PP2	D02	X_15	X_15F	acctttggaagagcagttggtg	X_15R	tccttcttggattgcaggtaac
PP2	E02	X_16	X_16F	tgactgcctttacctcccaagg	X_16R	ggctagaagtatttagctcagtgg
PP2	F02	X_17	X_17F	tggctggaggtaacatctgagc	X_17R	ggaatcaccatgtggttgttgg
PP2	G02	X_18	X_18F	tggaatggtttacaggctttgg	X_18R	ttaaaggcgtgtgccaccactg
PP2	H02	X_19	X_19F	actgaggtagtcggagctgacc	X_19R	acggcacagtttactcctgagg
PP2	A03	X_20	X_20F	gtcctctgatgtcaacatgtgc	X_20R	acgctctgcttctgccttcagg
PP2	B03	X_21	X_21F	agtgagactctgcacccatctc	X_21R	agtggaagcaaacaggcaatcc
PP2	C03	X_23	X_23F	cacccacatcatggtagctcac	X_23R	agctggcgggagactggtttgg
PP2	D03	X_24	X_24F	tgatgccctcttctggagtgtc	X_24R	aggcaaacacacgatttgctgc
PP2	E03	X_26	X_26F	gggattggttctcgtgtcaggt	X_26R	acagagtgagttccaggacagc
PP2	F03	X_27	X_27F	tgaagcacctttctgactgagc	X_27R	tgagcaccataacaagccatcc
PP2	G03	X_29	X_29F	tgcctggggtgagagcaccctc	X_29R	gagagctttgtgcgtgtgtgtg
PP2	H03	X_30	X_30F	tctgaacctgtaagccagctcc	X_30R	tccaggactttcagggctacac
PP2	A04	X_32	X_32F	ttgtggctcacaaccagcaacc	X_32R	agacagcccttgagcccagcag
PP2	B04	X_34	X_34F	acaactggagtggcagctatcc	X_34R	actgagtgaaccaccaatgctg
PP2	C04	X_35	X_35F	tgtgcttaggaaaccttgtgtg	X_35R	acgctgattggcttcccagcag
PP2	D04	X_36	X_36F	tctttcctaaggtccagtggga	X_36R	gctcctaactcatcaacacctg
PP2	E04	X_37	X_37F	accaagacgtgaggcaatggag	X_37R	agagaggcattaccttagcctc
PP2	F04	X_38	X_38F	gagtcactgttaaggtcgccct	X_38R	tgggagtgttcaggatcaggtg
PP2	G04	X_39	X_39F	actggaggtcttcacagccttg	X_39R	cagtctctgagttctagggcag
PP2	H04	X_42	X_42F	tgggagttgtagttccagggct	X_42R	atcacagtgcaggcttcatgga
PP2	A05	X_43	X_43F	acgtctctgctcatcctgggag	X_43R	tctgagccatctcttcagccag
PP2	B05	X_44	X_44F	aaccaaaccaggatgtcccagc	X_44R	taagccttccgaactcaactgc
PP2	C05	X_45	X_45F	aaggacttggatattgctccac	X_45R	cctgaactcacagagatccgtc
PP2	D05	X_46	X_46F	tcccacggcaaaggcaccaagc	X_46R	agctctttgagatacagccagg
PP2	E05	X_47	X_47F	acactttgtccatcagcattgg	X_47R	tgtcattccagtttgggtgagg
PP2	F05	X_50	X_50F	ccttccaggattccactgagag	X_50R	ttccagaagacccaaagccctc
PP2	G05	X_51	X_51F	tagctgcccacagtggatgtcc	X_51R	aaaccactggccgggaatgctc
PP2	H05	X_52	X_52F	tgatgccagatggaacccttgg	X_52R	tgcggttcacagaccagtcagc
PP2	A06	X_55	X_55F	acactcccaatcctccaagtgc	X_55R	tgcttgcaattcctgaaccagg
PP2	B06	X_56	X_56F	agcagggaaaggtgcagacctc	X_56R	agaactgggtaacaggagccac
PP2	C06	X_57	X_57F	aggtttcctctgacatctgcac	X_57R	gttgcctacaagggcacatgac
PP2	D06	X_58	X_58F	tgcctctgactcccaagagctg	X_58R	agcttggtctggagggagttcc
PP2	E06	X_59	X_59F	atgtgttccctggtcagagctg	X_59R	gaggtgagtggacagatggctg
PP2	F06	X_60	X_60F	atgtacactttggtggtcatgg	X_60R	agctctttggttccccccagag
PP2	G06	X_61	X_61F	aatgcttgcccagcttgtgtgg	X_61R	tgaagttgctttggtcagggtg
PP2	H06	X_62	X_62F	agcactgggagtatgcttgctg	X_62R	tgctgtgtgtggtgatgcatgg
PP2	A07	X_63	X_63F	gcacactcatcaaaccacagac	X_63R	agagaaactggtgtcatcctgc
PP2	B07	X_66	X_66F	cgggtcgaggctgctcacagtg	X_66R	cactctgtcttgagcctagctc
PP2	C07	X_67	X_67F	acttgtgcctgaggacttgagc	X_67R	cttgggaactttgcaggtgctc
PP2	D07	X_69	X_69F	atcctggactgtcaagccaagc	X_69R	tgccagggccaggaagcaggag
PP2	E07	X_71	X_71F	ttagtgggttgaatggacttgc	X_71R	agagtcctacaagtcctcaagg

PP2	F07	X_72	X_72F	tgtatgtgccaggtgtcctcag	X_72R	tctgcaagtccattcaacccac
PP2	G07	X_75	X_75F	tctctgactgctacacacacac	X_75R	aggtaaaggcatttgcagccag
PP2	H07	X_76	X_76F	taagggacttaggggcaaactc	X_76R	tagggattgaacccaaggttcc
PP2	A08	X_78	X_78F	agattccactctacggactagac	X_78R	ctgagtgaagtatccgaggctg
PP2	B08	X_79	X_79F	aaggctgagcatgagagtccac	X_79R	agaagatggcctccactactgc
PP2	C08	X_80	X_80F	ggtcttgtgataactgcctgtg	X_80R	tttccagcaaacacacacacac
PP2	D08	X_82	X_82F	ttcagggttcaaggtcagtctg	X_82R	acacagtgagttccatgccagc
PP2	E08	X_83	X_83F	attgtggctcaaaacactggtc	X_83R	ccatcagtggaagtctgagtgg
PP2	F08	X_84	X_84F	ttaggttgccagcacccatagc	X_84R	gatttgcctctaagtgcgttgc
PP2	G08	X_85	X_85F	agctgtgttcccttggaccagc	X_85R	tgatggttgcccatcccaggag
PP2	H08	X_86	X_86F	agcgttagccattgttggacac	X_86R	agcttgattatatggagcccag
PP2	A09	X_87	X_87F	agttgcccagctattgtgctgc	X_87R	tccctccctgaagtcaccaagg
PP2	B09	X_88	X_88F	aactcaggtgaagcacatttgc	X_88R	aatcccagtccgtggaaactgg
PP2	C09	X_90	X_90F	gcatctgcacaggtacacgcac	X_90R	agtgattgcgtatctctgccag
PP2	D09	X_91	X_91F	tgctccctgattggtctcagtg	X_91R	actgcacacgtcttggtccagg
PP2	E09	X_92	X_92F	caacatgcacccatgcacacac	X_92R	ttacgcttgaatgtgtacgagc
PP2	F09	X_93	X_93F	aggtctgatccttagggctcac	X_93R	atatagcaagttctgaggcagc
PP2	G09	X_94	X_94F	tgagaattgaacccagggcttc	X_94R	ggaagctacatactgacaagcac
PP2	H09	X_96	X_96F	agcaagttcagtggagctttgg	X_96R	tgctcagatcagtgtcagccac
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PP2	A11	X_105	X_105F	acactttgtccatcagcattgg	X_105R	tgtcattccagtttgggtgagg
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PP2	C11	X_107	X_107F	cacctacagagaccacacacac	X_107R	tgagtgcccacccagcccagca
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PP2	E11	X_112	X_112F	tgaaagcggattacccatagca	X_112R	tcccttgctctacacacacac
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PP2	A12	X_119	X_119F	cctatgtcccttccactcactc	X_119R	tcctgtctgaagtggtcagctc
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PP4	C7	Chr11_73	Chr11_73F	agaagctgtctctgcagcacac	Chr11_73R	agctgttcttctgtgactggtc
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PP6	A1	Chr01_1	Chr01_1F	tgggtgccctggagtctggagc	Chr01_1R	aggacgttgtagcgccgagtcc
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PP6	D1	Chr01_4	Chr01_4F	ggacacacttgtgctgtggtgc	Chr01_4R	ctgggtggcagtttacgaccac
PP6	E1	Chr01_5	Chr01_5F	agaccatgacactgggagcctc	Chr01_5R	ggaacatggttctgcaaggcca
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PP6	C2	Chr01_11	Chr01_11F	catgaacacgtgatgccaacag	Chr01_11R	tggagctgtcatgatggcacac
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PP10 E11	Chr04_31	Chr04_31F	tgaataatgtggcactgacctg	Chr04_31R	agagataaggccagccattctg
PP10 F11	Chr04_32	Chr04_32F	acagagtccacggaggctttgg	Chr04_32R	agaatgccttactcagaaccca
PP10 G11	Chr04_33	Chr04_33F	tgtgggtgtgcgagcatgtacc	Chr04_33R	agcctgatgcagtggctcacag
PP10 H11	Chr04_34	Chr04_34F	atgtaggcaaatacccatgcac	Chr04_34R	tgactcccagatgtgtgctgtg
PP10 A12	Chr04_35	Chr04_35F	tcccagagccagagcttccagc	Chr04_35R	tcctgggatctccgaagtctgg
PP10 B12	Chr04_36	Chr04_36F	agacctgagttcaggattcagc	Chr04_36R	tgtacttgcacctcctgggcac
PP10 C12	Chr04_37	Chr04_37F	tggcctcgaactcagagacctg	Chr04_37R	tgcctaaggacacacatgcacg
PP10 D12	Chr04_38	Chr04_38F	tgcaagctggcctgttgactgg	Chr04_38R	aaggttagctttcgtagtgcag
PP10 E12	Chr04_39	Chr04_39F	agaaacggatgatgcagttgtc	Chr04_39R	ttagtgatccaaagcccaggtg
PP10 F12	Chr04_40	Chr04_40F	ctgtgggttgaaggccattctg	Chr04_40R	caacgagtccttcgtcacgtgc
PP10 G12	Chr04_41	Chr04_41F	aggacccaggtttggttcccag	Chr04_41R	agtcacacaccactacacctgg
PP10 H12	Chr04_42	Chr04_42F	aaaccaccagccacttggcctg	Chr04_42R	ttcgtgtgtcacgtgtccaagg

Supplement 3 Primer sequences of the microsatellite loci genotyped along the candidate regions.

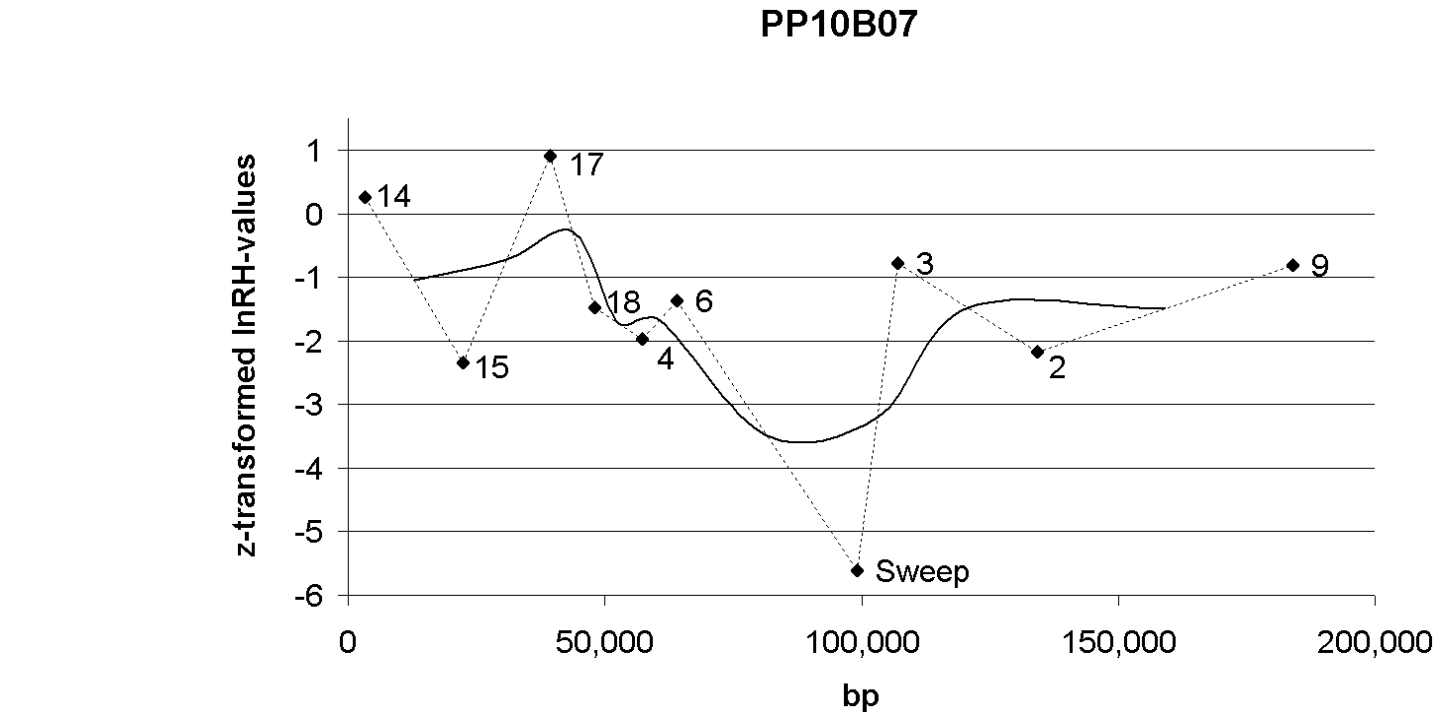
Markername	Primer	Sequence	Primer's Name	Sequence
PP9A07_12	PP9A07_12_F	tgccacgggaaccctctcggtg	PP9A07_12_R	tggctttcagtacactggacag
PP9A07_14	PP9A07_14_F	aggctgacgcacatggcggctg	PP9A07_14_R	tcctgaagccagacctcctgag
PP9A07_16	PP9A07_16_F	gtggaccgtgatgcaccttagc	PP9A07_16_R	ccagtcagtctagcttaggcag
PP9A07_17	PP9A07_17_F	acaacacagatggacatccctg	PP9A07_17_R	tcacttggcttacagaagtgct
PP9A07_18	PP9A07_18_F	agccatcagcttacttgagcag	PP9A07_18_R	cctgggatctgctgtgtgatgg
PP9A07_3	PP9A07_3_F	tcaaggtccagggctgagtctg	PP9A07_3_R	atgcaagcctgttgacctgagc
PP9A07_4	PP9A07_4_F	agacctccaaagcccggatcag	PP9A07_4_R	tgaccaccagccaaggaccctg
PP9A07_5	PP9A07_5_F	agccttctgtctgcctgagtgc	PP9A07_5_R	cagattcagctggactggctgg
PP9A07_6	PP9A07_6_F	atggtcgctgggcagagctgtc	PP9A07_6_R	cactaggccatgtctgcaaacc
PP9A07_8	PP9A07_8_F	ttactaagcctgccttgagagg	PP9A07_8_R	caaagattgtgtcctgggaacc
PP9A07_9	PP9A07_9_F	accgatggcttgagttcggtcc	PP9A07_9_R	tttggttgcacatatgcgtgtg
PP9A07_10	PP9A07_10_F	agattgtggtcttggtttctgg	PP9A07_10_R	aggaaatggcttccaccatacc
PP10B07_14	PP10B07_14_F	tgtctccaggcactggcatagg	PP10B07_14_R	tggatgcctgcgcacctagcag
PP10B07_15	PP10B07_15_F	tggcttgactacacatgcacac	PP10B07_15_R	ggcctcagacaggcagcaatcc
PP10B07_17	PP10B07_17_F	acttcgatgctttccgtcagtg	PP10B07_17_R	tgtcaggcatctcccaactgtg
PP10B07_18	PP10B07_18_F	tgtcaggctttagctgagttgg	PP10B07_18_R	agctgactgaggccgtggtgtc
PP10B07_4	PP10B07_4_F	agccttgggtcaggctcagtgg	PP10B07_4_R	tgtgcagagcattgatggctgc
PP10B07_6	PP10B07_6_F	accagggcagtcacagtgttcc	PP10B07_6_R	actgggcatttgtgtgtccgtg
PP10B07_3	PP10B07_3_F	atagacgtgctctgcatgactg	PP10B07_3_R	aggagccttgtgactacagcca
PP10B07_2	PP10B07_2_F	acctgccttgtaggcctgggac	PP10B07_2_R	tggtcctgaggcaccaaccctg
PP10B07_9	PP10B07_9_F	aaactcagggctttgggcgtgc	PP10B07_9_R	tctggacctgcttccaacacac
>PP7F04_5	PP7F04_5_F	agacacctggattgtcctctgg	PP7F04_5_R	tgtagactcacagggcacctgg
>PP7F04_7	PP7F04_7_F	gttgctatgggcatgcttgcac	PP7F04_7_R	agatgtcacccagcacacctgg
PP7F04_2	PP7F04_2_F	accaggagtccaaggctaacct	PP7F04_2_R	ccaaatccacacaagcacgcac
PP7F04_3	PP7F04_3_F	ttgttgtctagtgtacggttgg	PP7F04_3_R	aggctgacaaacacctgcaagc
PP7F04_4	PP7F04_4_F	tacctctgtgtgcacgtgagtg	PP7F04_4_R	ggaaggagctatgggtccacct
PP5F12_3	PP5F12_3_F	aggcaccagtaggtgcacatcc	PP5F12_3_R	tccagacaaggcagtgcaagct
PP5F12_4neu	PP5F12_4neu_F	tgcagatggacagccagcatcg	PP5F12_4neu_R	acacactgagcaggttgcgttc
PP5F12_8	PP5F12_8_F	ctggcaagactcagcctcacag	PP5F12_8_R	gtccagacaatccctcacagac
PP5F12_5	PP5F12_5_F	cttaacgaagtttccagtggct	PP5F12_5_R	caagatgctgaaacacacacac
PP5F12_6	PP5F12_6_F	ccacaagcatccgagaaccctg	PP5F12_6_R	tgccatcaagccacatccaagc
PP5F12_7	PP5F12_7_F	agtaaggtgggaaatgtggacc	PP5F12_7_R	ggccagcctgctctacatggca
PP8D01_18	PP8D01_18_F	tgtggtgtacctgctacacgag	PP8D01_18_R	tgccatagtccaggcctctgtg
PP8D01_20	PP8D01_20_F	agccaagtaccaagtgctcgtc	PP8D01_20_R	gcagtgagtttcatggtagcac
PP8D01_7	PP8D01_7_F	tgtgggacctccagacaccctg	PP8D01_7_R	tgaggcccgcttgcctacagag
PP8D01_1	PP8D01_1_F	agatgccttgcaaccacttgtg	PP8D01_1_R	gagacactgcctacctggcagc
PP8D01_2	PP8D01_2_F	gttggccacctaggaactggtc	PP8D01_2_R	aattcccagcaaccacatggtg
PP8D01_3	PP8D01_3_F	aggttcacacacatgacagctc	PP8D01_3_R	agcaccacctggctgagtccag
PP8D01_4	PP8D01_4_F	ttgttgttgctaggttggttgg	PP8D01_4_R	tgagcttgataccagcttggtc
PP8D01_10	PP8D01_10_F	tcagtttgctgttagccacgag	PP8D01_10_R	tcaccattgctagccatcagag
PP8D01_11	PP8D01_11_F	tcagatcccacacacggaggtc	PP8D01_11_R	agtgggagacctgagcctgtcc

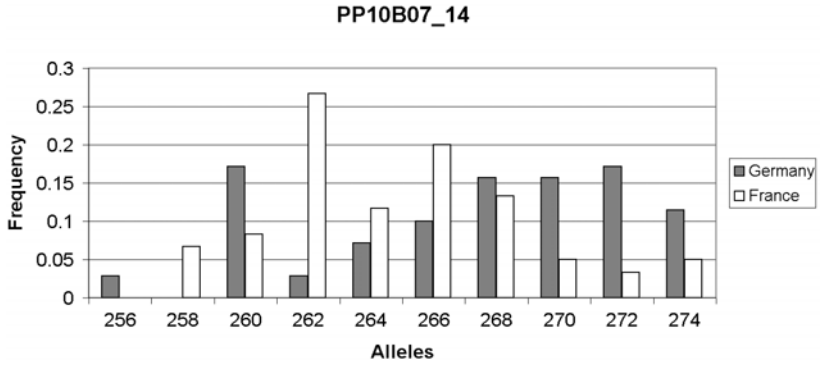
PP8D01_12	PP8D01_12_F	aagccgagtgtggtgttcatgc	PP8D01_12_R	aagccttaggaccaccgctcag
PP8D01_15	PP8D01_15_F	cagattgagttccaggacaacc	PP8D01_15_R	agttgccctgttgtggatgtgc
PP8D01_16	PP8D01_16_F	tgcaccgcacaaatgccacctg	PP8D01_16_R	ggattgacacatggcccactgg
PP4H12_8	PP4H12_8_F	actctgtgacctcccaggttag	PP4H12_8_R	ggccacaagccagccaagtctg
PP4H12_1	PP4H12_1_F	tgcatcctaccaagcaagccac	PP4H12_1_R	cccaggccaacttgcgttacag
PP4H12_10	PP4H12_10_F	ctagccctggagagtgctgacc	PP4H12_10_R	gaagttccctttggcttgctgg
PP4H12_3	PP4H12_3_F	tgggtgttgccctaagcccagg	PP4H12_3_R	agcaagcctgtgctcacacagc
PP4H12_12	PP4H12_12_F	aatgggcatgtttcaagtgtgc	PP4H12_12_R	ctgctgtgacctggctggaacc
PP4H12_2	PP4H12_2_F	accttcaagactggatctggtg	PP4H12_2_R	tttccagcctgttgggtttagc
PP4H12_5	PP4H12_5_F	agggccaagaagtgggagtctg	PP4H12_5_R	tcaggactctacagcagtggct
PP4H12_4	PP4H12_4_F	cctcacaggaagtcatcacagc	PP4H12_4_R	acgcctttaatcccagcactcg
PP3H03_2	PP3H03_2_F	tctaggacggccagggctacac	PP3H03_2_R	acggttgctatggcactgttgc
PP3H03_3	PP3H03_3_F	tgccaggacattcctgagcacc	PP3H03_3_R	agaccctgagccctgggaccac
D10Mit23	D10Mit23_F	tgagccctgccgtgtgcctcag	D10Mit23_F	caagccctaggttcccatgagc
PP3H03_5	PP3H03_5_F	tgctgaaggacctccacattgg	PP3H03_5_R	agctcctcctgcctcccaagag
PP3H03_6	PP3H03_6_F	attgtcctgttgggaaccttgg	PP3H03_6_R	aggtctcaaggctgggctgctg
PP3H03_7	PP3H03_7_F	cattcacaggatccatgtccag	PP3H03_7_R	aggcaacatggtctccggaacc
D1Mit305	D1Mit305_F	gtgggaaccttctactatttctatgc	D1Mit305_R	gtgtacctcctttctgtttatggg
PP6E03_1	PP6E03_1_F	agaccaatgtctttggacacag	PP6E03_1_R	gcacttcccaacacaaaggtcc
PP6E03_3neu	PP6E03_3neu_F	ccttgcttgcagagagtcccac	PP6E03_3neu_R	tgggcattgaaacctcagaacc
PP6E03_2	PP6E03_2_F	ggacagacacctgaggttgtcc	PP6E03_2_R	gacagacacctactctgtgtgg
D1Mit440	D1Mit440_F	tccacacaaggtgtcctctg	D1Mit440_R	gctcaggtgacctccaaaac
PP6E03_5	PP6E03_5_F	cacccattgacagctccaagac	PP6E03_5_R	ccacatgatggttcacaaccac
PP6E03_6	PP6E03_6_F	tgtcacatgggctcccgcaagc	PP6E03_6_R	tctggatggtctagcaagcacc
PP6E03_7	PP6E03_7_F	atgccaaaccttagctctggag	PP6E03_7_R	acactttgacaccgacccacag
PP6E03_9	PP6E03_9_F	acactggactagacccagaacg	PP6E03_9_R	agactgttgtgcacctctgtgg

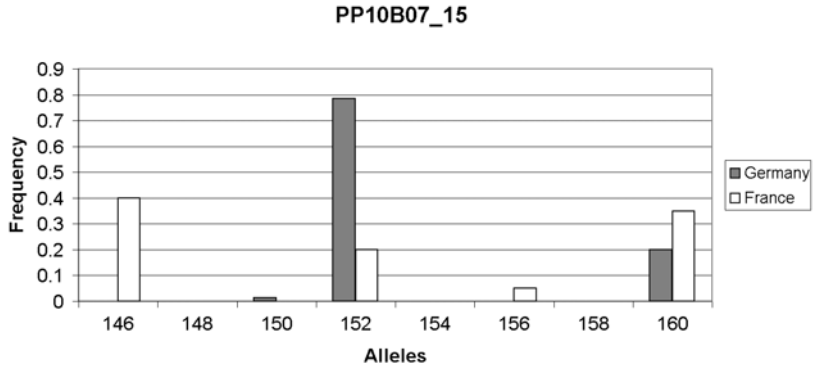
Supplement 4 Primer sequences of the loci genotyped along the 'neutral regions'.

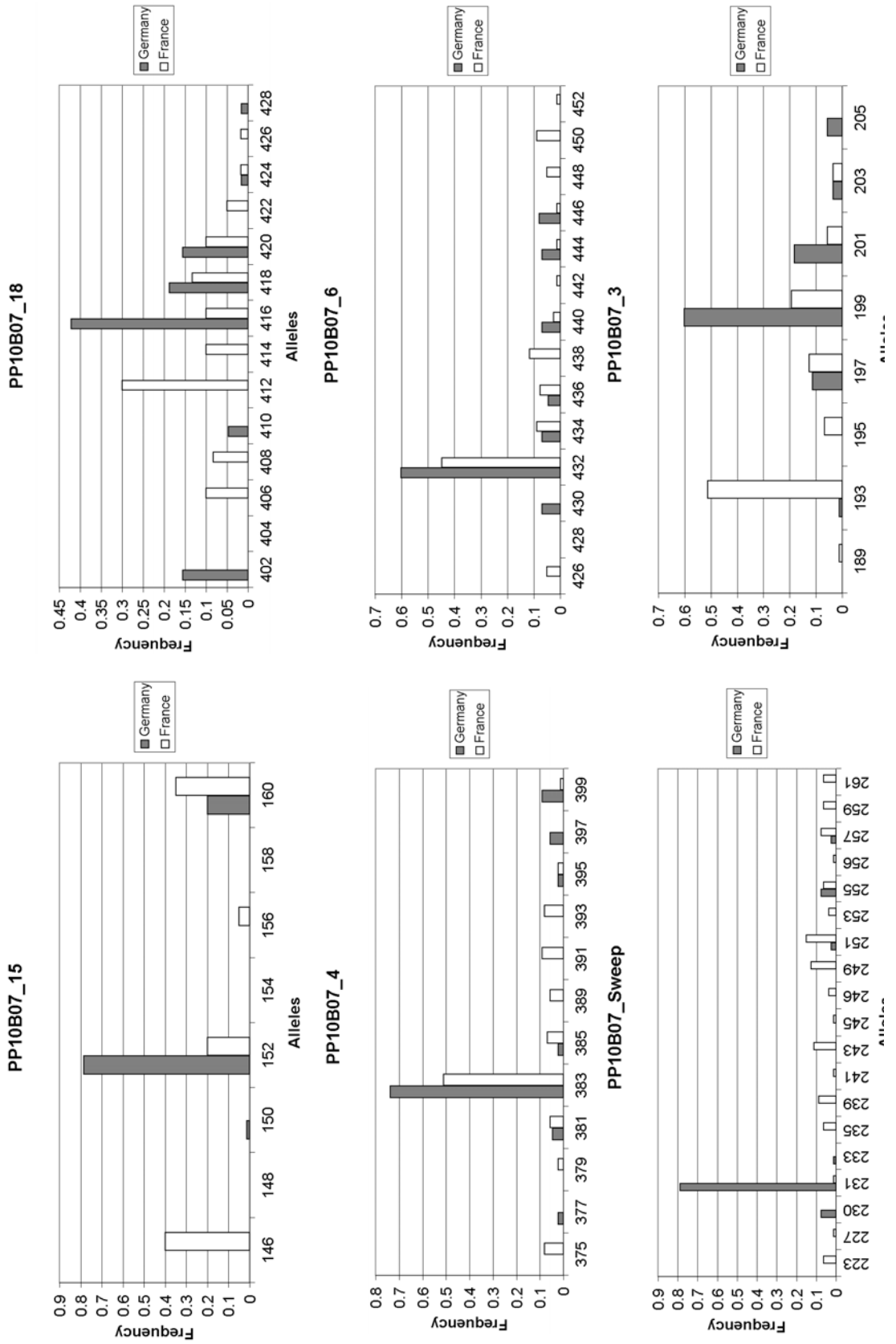
Marker	Chr	Primer's Name	Sequence	Primer's Name	Sequence
N1_01	3	N1_1F	agcaagaattgttcccgaatgc	N1_1R	aggcagttggagcagcttgagg
N1_02	3	N1_2F	gcctgcagccatccaaagctgg	N1_2R	ccagttcagagcaggcatttgg
N1_03	3	N1_3F	tctctgctgggagcacactcag	N1_3R	acccagcacttgggtcttcagg
N1_04	3	N1_4F	accagatcgcaggagatggctc	N1_4R	agagggttgtatgcctcctgag
N1_05	3	N1_5F	tcctgatgtctgccaaccagag	N1_5R	agagcggcagccatgccttcga
N1_07	3	N1_7F	tcagccttggtgagagcacctg	N1_7R	agcaggaagttagctctgtagc
N1_10	3	N1_10F	accacgcacacaaacaagcctc	N1_10R	agggagcactatccttgcagtg
N1_11	3	N1_11F	agggcagctttggctacagacc	N1_11R	tgactgaccaaccagcaaccag
N2_01	6	N2_1F	acccagattgttgagagccacc	N2_1R	tgtctttaggggacggacacac
N2_04	6	N2_4F	agtatgctgcttgatgctcctg	N2_4R	tctcaaggcacacagcacctgc
N2_05	6	N2_5F	aacaagttatgcccacacaagg	N2_5R	tgacttccacacaatcagcctg
N2_11	6	N2_11F	aggtgtgtaccaccatacgtgg	N2_11R	tgcaaaactgtagccgacatgg
N3_01	8	N3_1F	tccagacctcagccttgaccag	N3_1R	tgtgttgagtcaggatctgtgg
N3_02	8	N3_2F	aaccttggtccagccttagagg	N3_2R	tgagagcctattcagggttagc
N3_04	8	N3_4F	gaggactgactggtctagcacc	N3_4R	ggaatgtgtgtgtgcagatgac
N3_08	8	N3_8F	ggagatggctagatgtcgctgc	N3_8R	gctttgggaacagattccggtg
N3_09	8	N3_9F	tgttgccacatacccagcaaag	N3_9R	tcttgacaccggaggttccaca
N3_12	8	N3_12F	tcccactgatgtccaacaaagc	N3_12R	tggttccaaactgacagggctg
N4_01	17	N4_1F	tgtaccttgcctggatgcagag	N4_1R	gagcatggccttatctgacagc
N4_02	17	N4_2F	gtatgcagtggaaacacctgct	N4_2R	gaagcaagccttcctaagctgc
N4_06	17	N4_6F	ggttctgatagaggcatgttgtc	N4_6R	tgtcccaagttcagacacctgg
N4_07	17	N4_7F	ctataaagttgctgttgcccga	N4_7R	agtcaccagtcagatcaggcag
N4_08	17	N4_8F	tgggatagggcgactccagagg	N4_8R	tgcaaacccaatagacacctcc
N4_09	17	N4_9F	tgtattctatcccatgcccgag	N4_9R	gtgaacacacgcgcacacatgc
N4_13	17	N4_13F	actaggggacttgtacccatgc	N4_13R	tccaagctcacagttgcctgag
N4_15	17	N4_15F	aagctgctgattaccaccaaag	N4_15R	ctagggtcttctaccacaatgg
N4_18	17	N4_18F	tcaactgcctcaggggttgtcc	N4_18R	agctattgtagcccaactggct
N5_02	4	N5_2F	acgctgacaccattgcgtacac	N5_2R	catcttgtggagagagcggttc
N5_03	4	N5_3F	ttaatcttgcccatccatgagc	N5_3R	accttcctctacacacacagac
N5_04	4	N5_4F	tttgggtggaaagtccatgagc	N5_4R	aagacagctttctgcttgagac
N5_07	4	N5_7F	cccatcactggaggtctcatcg	N5_7R	gggcctagatgtcctgagagct
N6_03	12	N6_3F	accagcagcccaatcacagcag	N6_3R	taattccaagcagtggtactgg
N6_05	12	N6_5F	tgtaccaggatatcatgcaggt	N6_5R	tagagccttcaccacaacatgc
N6_08	12	N6_8F	ggtttctttgtgtccgtgaagg	N6_8R	caggtgtcagtgttcttgcctg
N6_09	12	N6_9F	tcatgcaaacacagccattgtc	N6_9R	cctaatccttctccaggagcac
N6_10	12	N6_10F	aggtgtccattaatgccctgtc	N6_10R	gggcatgtaataccttgaggac
N8_01	7	n8_1F	tccaagactttgtgggtgccag	N8_1R	agcaccatgtctgcctgaatgc
N8_02	7	n8_2F	accttaaggcatgcaaaccctg	n8_2R	tggtactgaccaaggccctttg
N8_03	7	n8_3F	agatgcacctaatcctccagag	n8_3R	agcctgcagaaagcagttctcc
N8_09	7	n8_9F	acatagatggttgtgatggtcc	n8_9R	ttcaggaccttggtcaggagtc
N8_13	7	n8_13F	atctggctttatgttgagggct	n8_13R	agtcctcagacctaggacccag
N8_19	7	n8_19F	tgaagaaacaagctggagtagc	n8_19R	ttaaaggcgtgtgccaccatgc
N9_05	11	n9_5F	acagcactcccgtggactcagg	n9_5R	agttagttcaggacatgcagtc
N9_08	11	n9_8F	ttcttgcgtgcctgctgaggac	n9_8R	aactggccacatggcctttacc
N9_13	11	n9_13F	tcaatgccattgtccagaggtc	n9_13R	aacttcggtgtttgctaaggca
N9_14	11	n9_14F	tccaagttcagatggacacacc	n9_14R	tctttgtgcatcagttccctgg
N9_16	11	n9_16F	tcctcctctgtgagctgcactc	n9_16R	gttgtttgcagatgtgtgtgtg
N9_17	11	n9_17F	ttcagtgtgcaagagtgcttgc	n9_17R	ggataggctcaacccatccagg

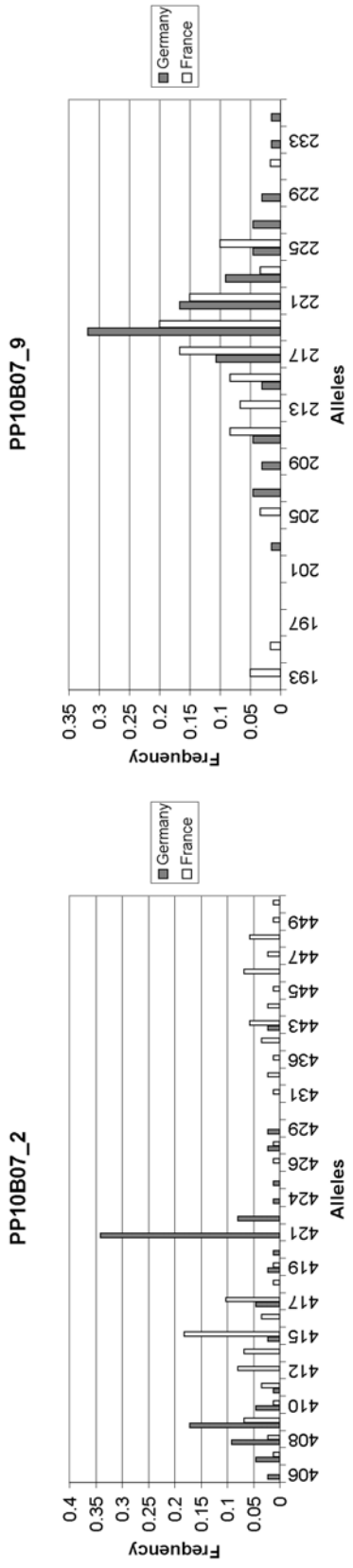
Supplement 5 Allele frequencies of the loci genotyped in candidate region PP10B07 (German sweep).

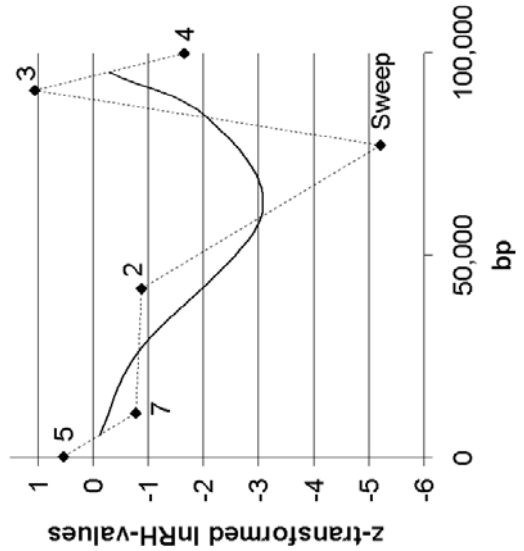




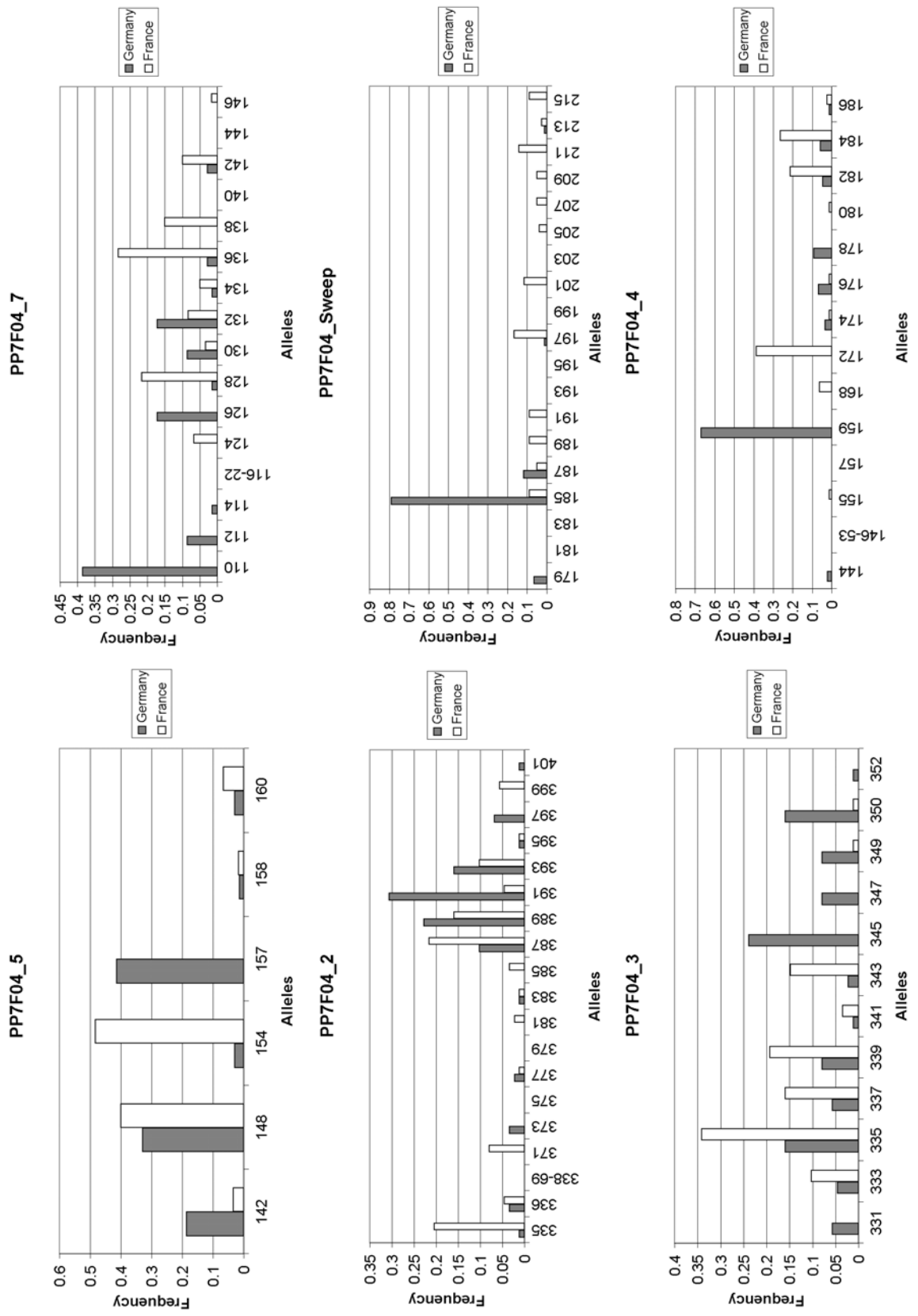



PP10B07_18
PP10B07_6
PP10B07_3
PP10B07_15
PP10B07_4
PP10B07_Sweep
Germany
France
Frequency
Alleles

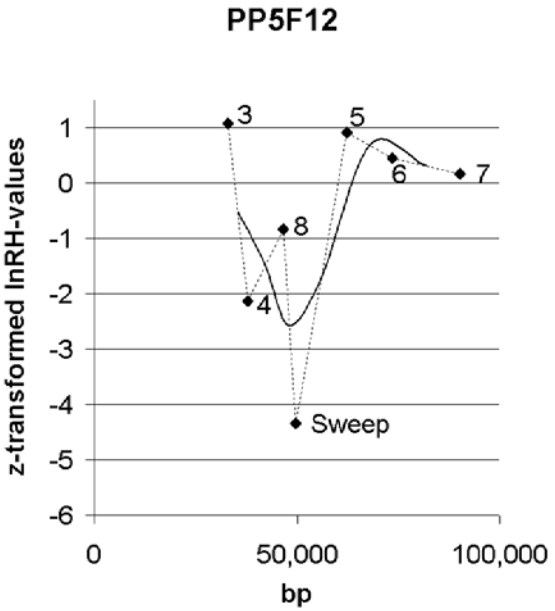


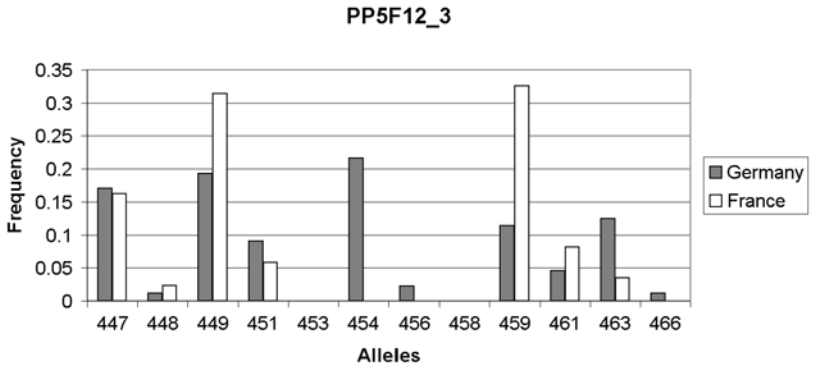


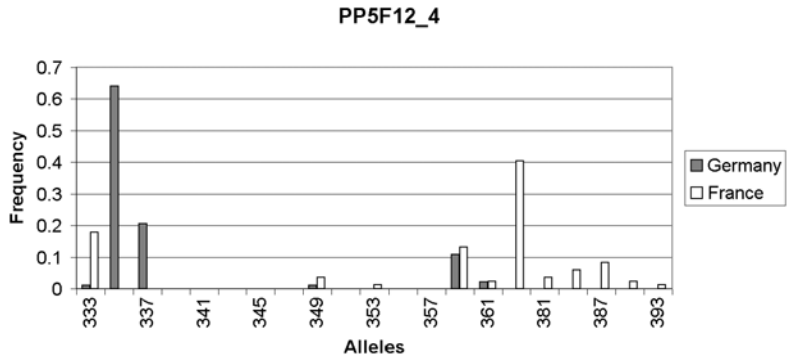
Supplement 6 Allele frequencies of the loci genotyped in candidate region PP7F04 (German sweep).

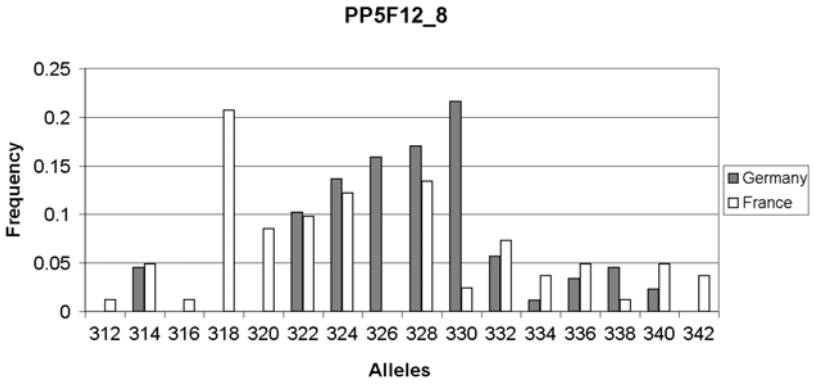

PP7F04_7
PP7F04_Sweep
PP7F04_4
PP7F04_5
PP7F04_2
PP7F04_3
Germany
France
Frequency
Alleles

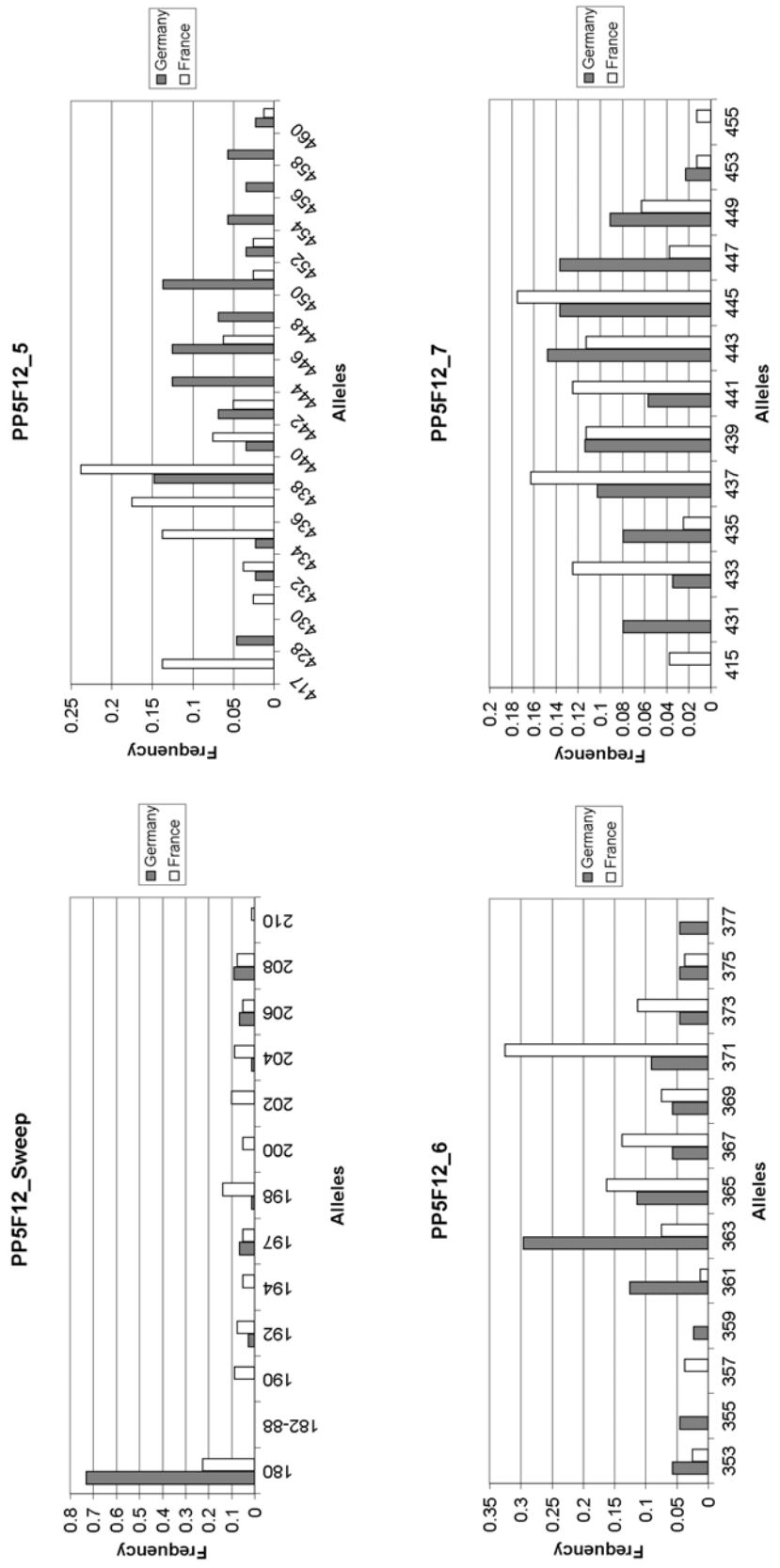
Supplement 7 Allele frequencies of the loci genotyped in candidate region PP5F12 (German sweep).

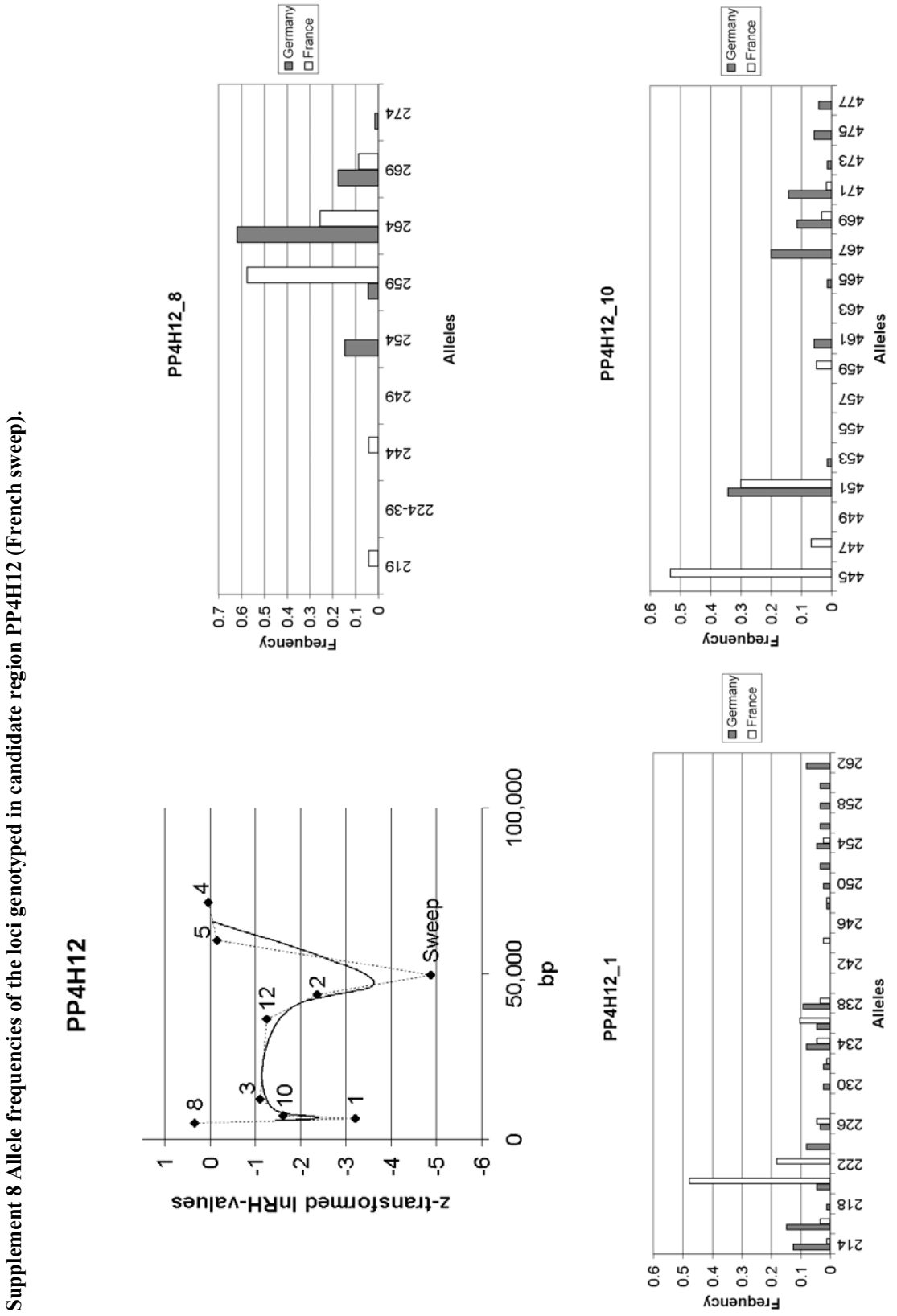




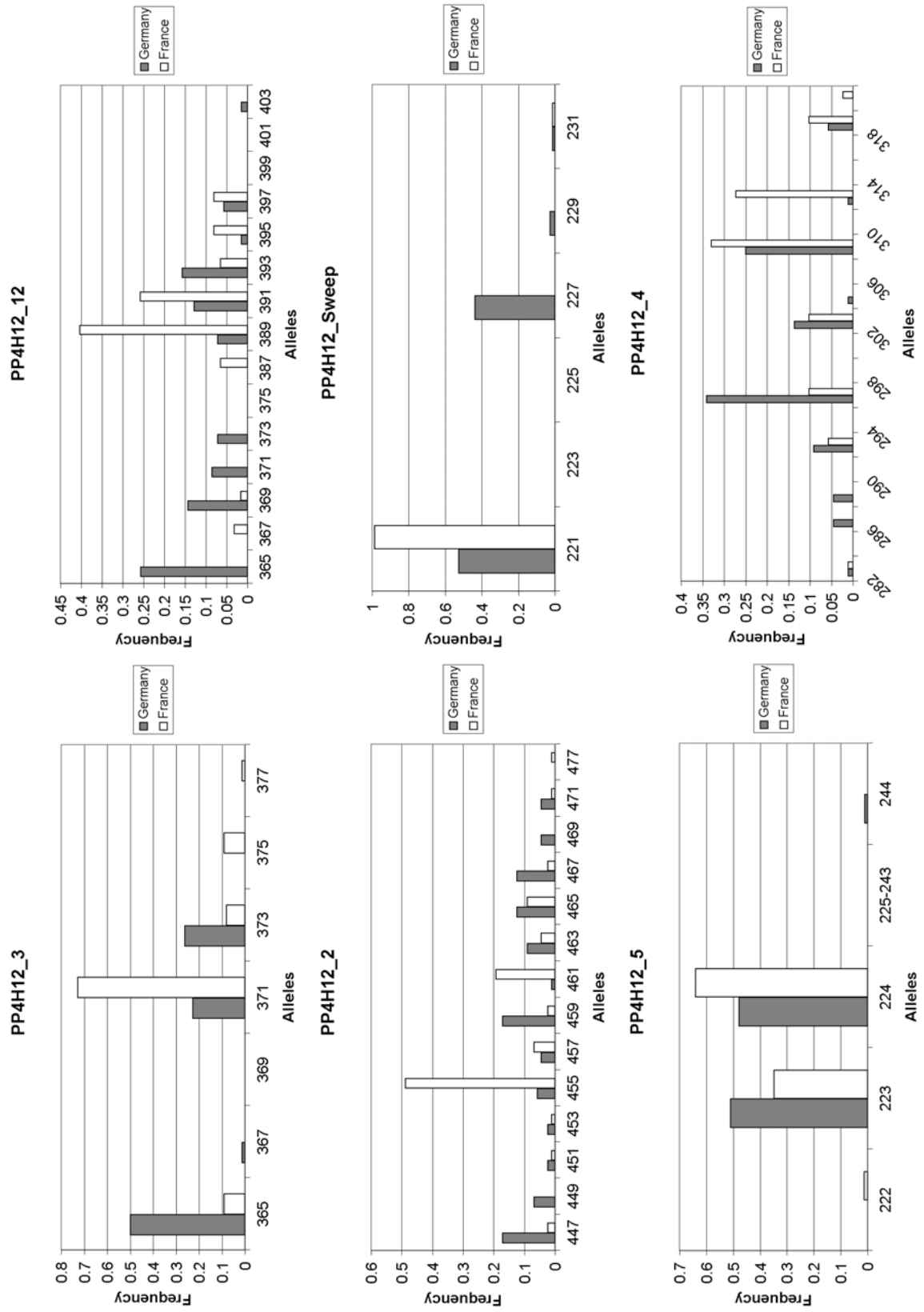




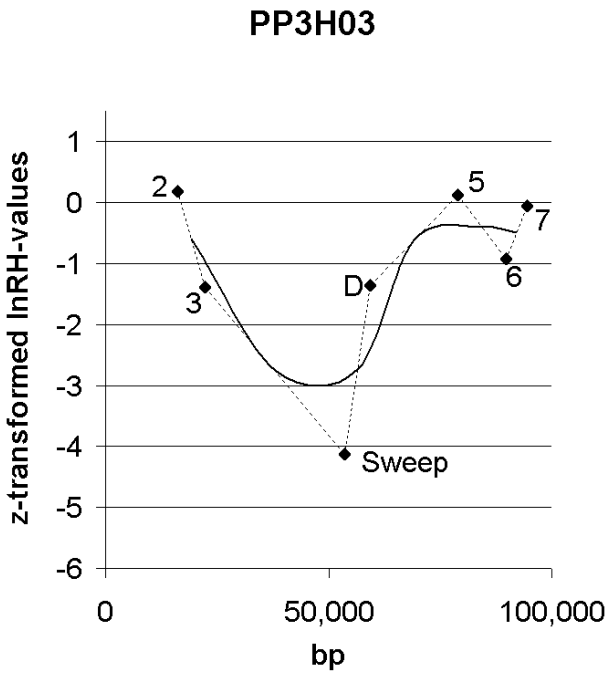

PP5F12_5
Germany
France
Frequency
Alleles
PP5F12_7
Germany
France
Frequency
Alleles
PP5F12_Sweep
Germany
France
Frequency
Alleles
PP5F12_6
Germany
France
Frequency
Alleles

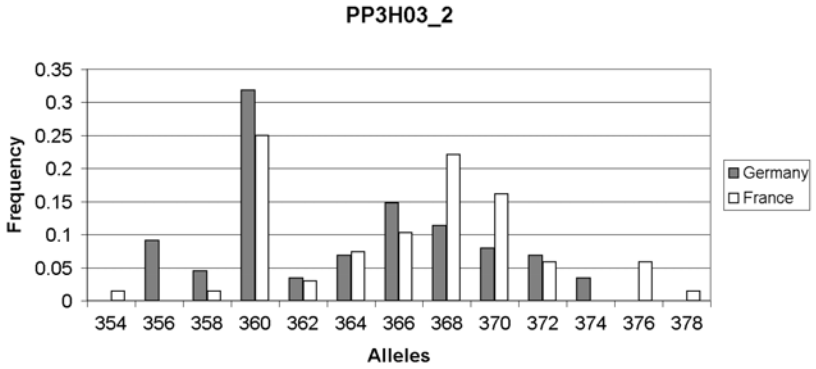


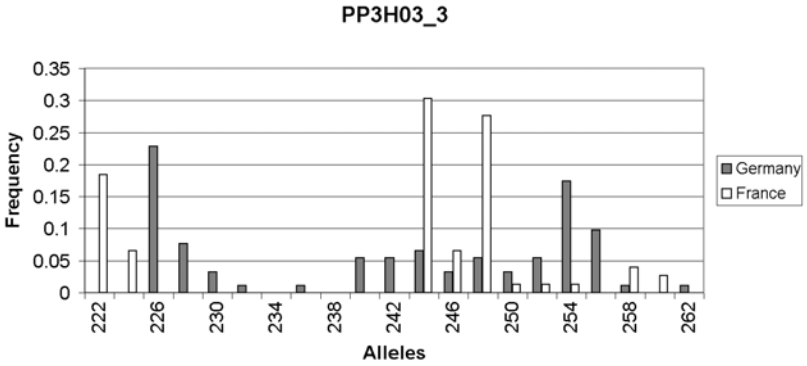
Supplement 8 Allele frequencies of the loci genotyped in candidate region PP4H12 (French sweep).

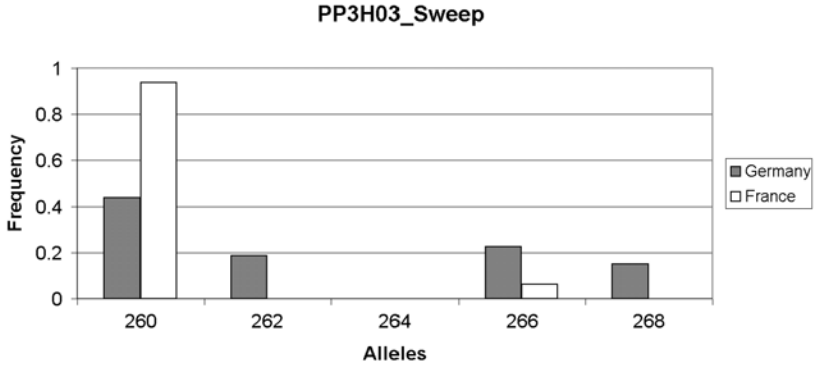

PP4H12_12
PP4H12_Sweep
PP4H12_4
PP4H12_3
PP4H12_2
PP4H12_5
Germany
France
Frequency
Alleles

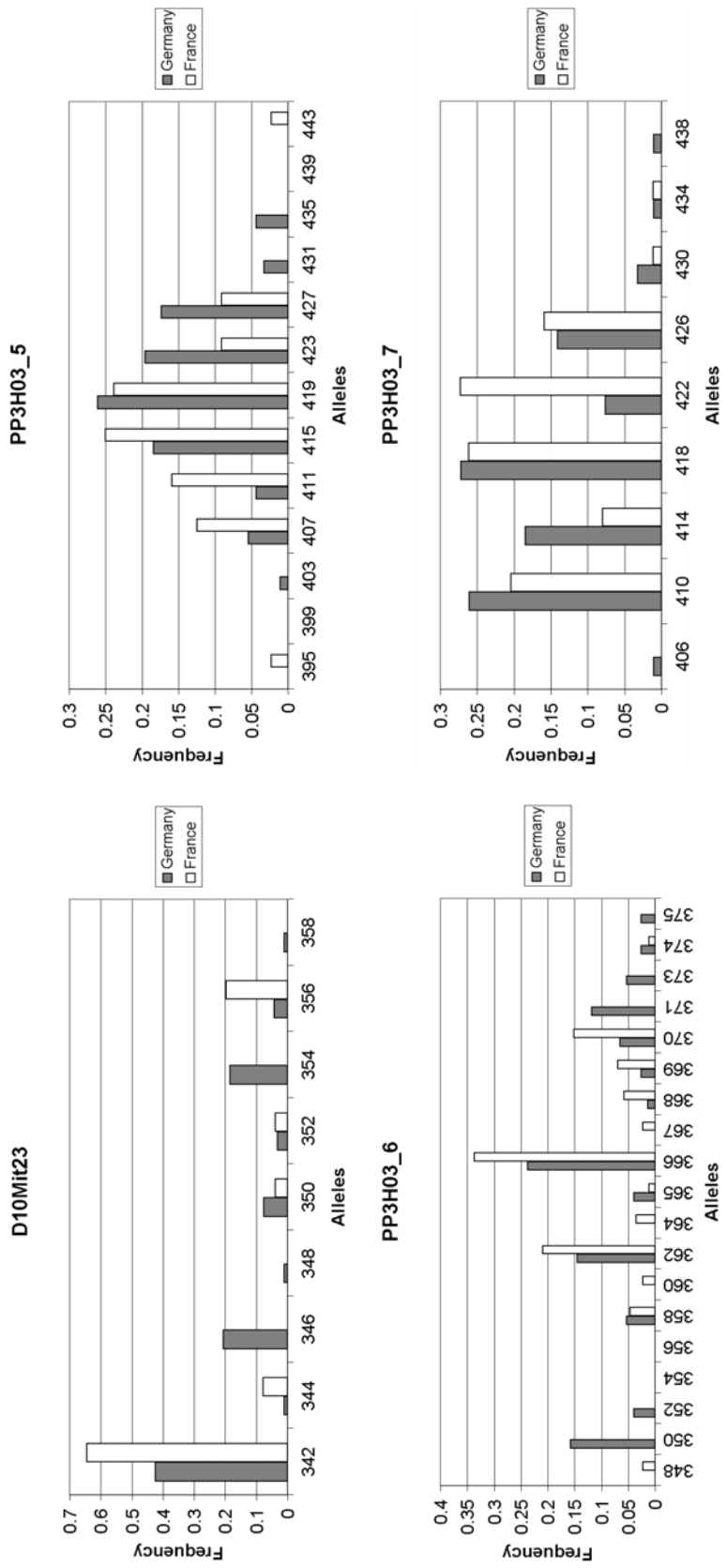
Supplement 9 Allele frequencies of the loci genotyped in candidate region PP3H03 (French sweep).



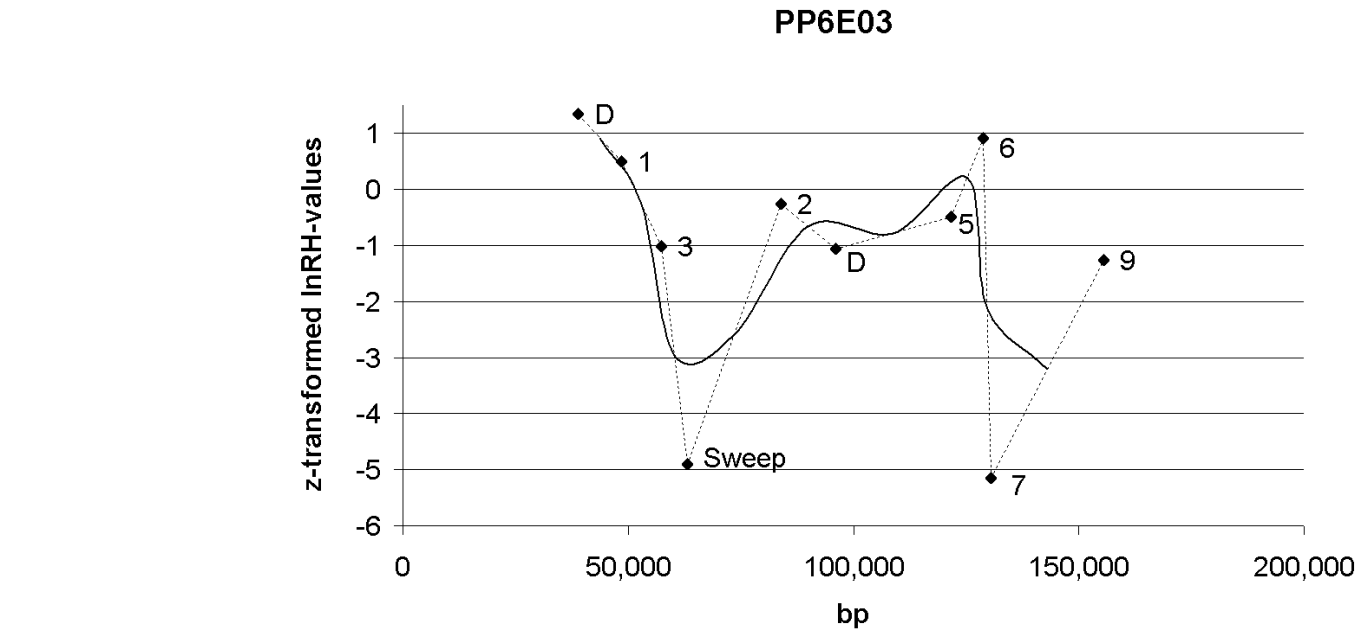


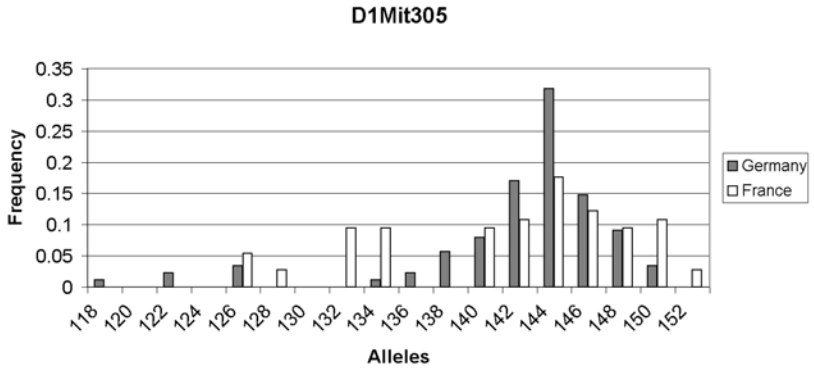


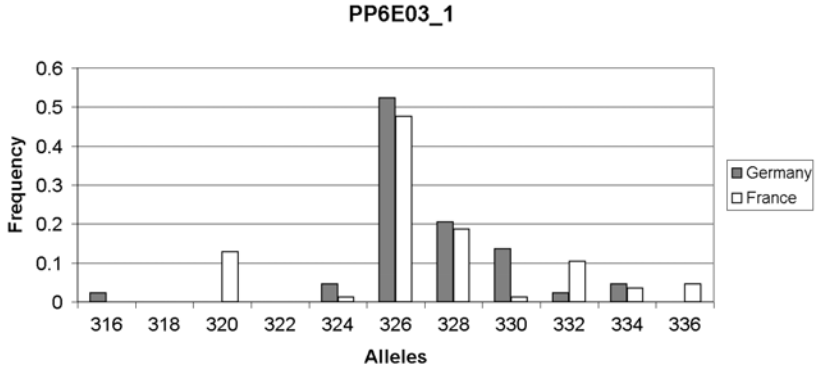


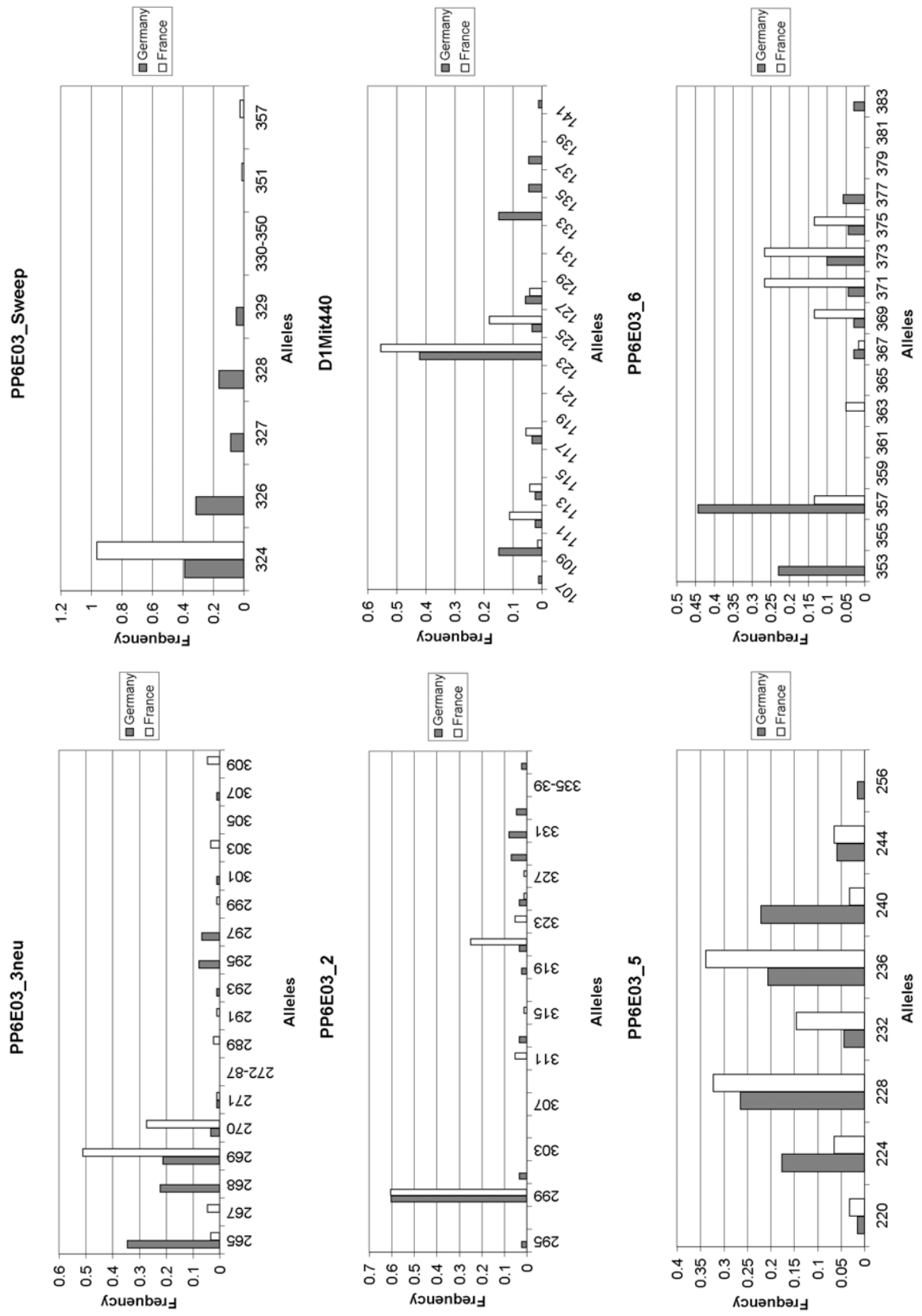

PP3H03_5
Germany
France
Frequency
Alleles
PP3H03_7
Germany
France
Frequency
Alleles
D10Mit23
Germany
France
Frequency
Alleles
PP3H03_6
Germany
France
Frequency
Alleles

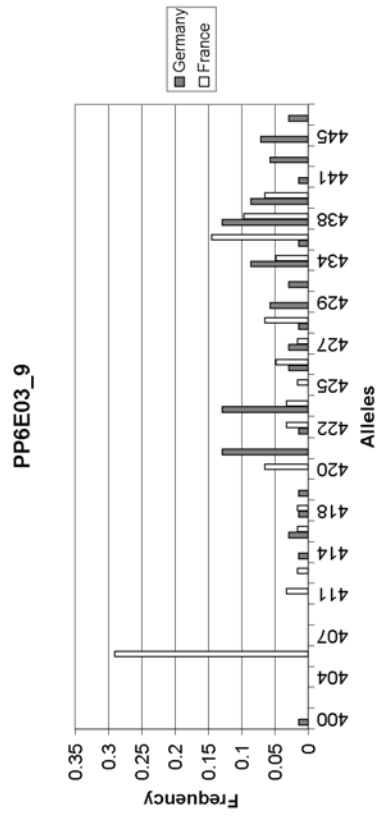
Supplement 10 Allele frequencies of the loci genotyped in candidate region PP6E03 (French sweep).

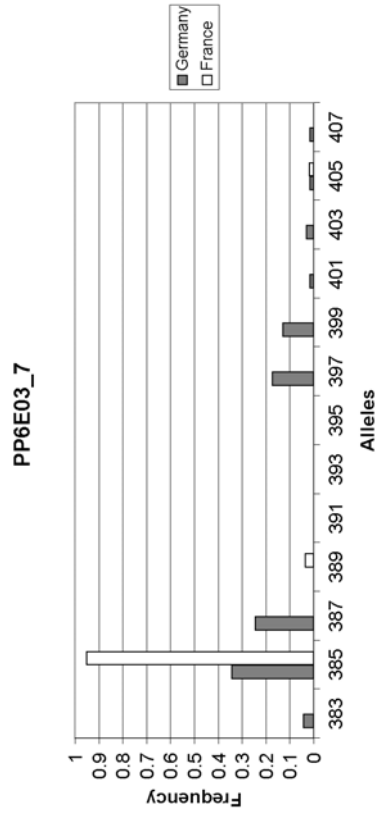







PP6E03_Sweep
D1Mit440
PP6E03_6
PP6E03_3neu
PP6E03_2
PP6E03_5
Germany
France
Frequency
Alleles


PP6E03_9
Germany
France
Frequency
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Alleles
400
404
407
411
414
418
420
422
425
427
429
434
438
441
445


PP6E03_7
Germany
France
Frequency
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Alleles
383
385
387
389
391
393
395
397
399
401
403
405
407

6 Digital Supplement

- PDF files of the pool pattern of all 960 microsatellite loci
- Row data of pool patterns
- PDF of a table containing the sequences of all amplified fragments

Erklärung

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Herrn Prof. Dr. Diethard Tautz betreut worden.

Köln, den 29.03.2006

Teilpublikationen:

Die folgende Publikation basiert auf Teilen dieser Arbeit und entspricht Kapitel 2:

Thomas, M, Ihle, S, Ravaoarimanana, Y, Kraechter, S, Wiehe, T, Tautz, D. 2005. Microsatellite variability in wild populations of the house mouse is not influenced by differences in chromosomal recombination rates. Biological Journal of the Linnean Society **84**: 629-635.

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