Effective population sizes and adaptive genetic variation in a captive bird population

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Abstract

Captive populations are considered a key component of *ex situ* conservation programs. Research on multiple taxa have shown the differential success of maintaining demographic versus genetic stability and viability in captive populations. In typical captive populations, usually founded by few or related individuals, genetic diversity can be lost and inbreeding can accumulate rapidly, calling into question their ultimate utility for release into the wild. Furthermore, domestication selection for survival in captive conditions is another concern. Therefore, it is crucial to understand the dynamics of population sizes, particularly the effective population size, and genetic diversity at non-neutral, at adaptive loci in captive populations.

In this study, we assessed effective population sizes and genetic variation at both neutral microsatellite markers, as well as SNP variants from the MHC-B locus of a captive Red Junglefowl population. This population is a rare instance of a well-documented history in captivity, and also following a realistic chain-of-custody, unlike captive lab populations. Our analysis of 27 individuals that comprise the entirety of one captive population. Our results show very low neutral and adaptive genetic variation, as well as low effective sizes, which are surprising in spite of the known demographic history. Finally, our study also shows the divergent impacts of small effective size and inbreeding in captive populations on microsatellite versus adaptive genetic variation in the MHC-B locus. Our study provides insights into the difficulties of maintaining adaptive genetic variation in small captive populations.

Introduction

Genetics of captive populations

The management of demographic security and genetic diversity are among the central considerations in conservation (1986a, b). Captive populations have long played a key role in conservation as a management tool to ensure demographic security (Hedrick 1992). The earliest work in conservation of endangered species focused on breeding in captivity to increase population sizes and intended for eventual release back into the wild. However demographic security and the maintenance of genetic diversity can sometimes be at odds, as genetic diversity is derived from the effective population size (N_e). One issue in captive populations is the differential reproductive success of some breeding pairs that are better suited to captive conditions, leading to selection for survival in captive environments. This phenomenon can further reduce the N_e due to founder effects (Nei *et al.* 1975; Newman & Pilson 1997). From the perspective of the reintroduction of the offspring of such captive breeders back into the wild, this may be a less than ideal situation, as these captive-adapted offspring may not carry the adaptive genetic variation required for survival in their natural habitat. Historically, captive individuals were raised for the genetic support of threatened wild populations, the genetic compatibility (prevent outbreeding depression) and genetic variation (ability to adapt to natural environment). However, these objectives can be materialized only if genetic diversity can be sustained, and if genetic drift and inbreeding can be limited.

The relationship between population bottlenecks and reduction in effective population sizes and genetic diversity has been reported from various vertebrate taxa (Athrey *et al.* 2012; Athrey *et al.* 2011; Emami-Khoyi *et al.* 2017; Leberg 1992; Lovatt & Hoelzel 2014). The loss of genetic

diversity due to drift and inbreeding in captive populations has also been well documented (Willoughby *et al.* 2015; Willoughby *et al.* 2017) and the drawbacks of using closed captive breeding populations for reintroduction have been described (Lynch & O'Hely 2001). These range from the rapid decline of fitness upon reintroduction (Araki *et al.* 2007)to a range of consequences for genetic diversity (Witzenberger & Hochkirch 2011). Witzenberger et al. (2011) also suggest the need for assessment of genetic diversity before *ex situ* conservation programs. The consensus of these studies suggest that considerations of genetic diversity of captive populations may be as crucial as maintaining demographic stability, but is perhaps much more challenging than the maintenance of demographic stability, because non-relative matings are not entirely avoidable. While some studies have focused on assessing genetic diversity, in many instances the history of the founding individuals is not well known, or the studbook data may be incomplete (Witzenberger & Hochkirch 2011). Additionally, inbreeding estimates may assume that founders are unrelated (Ruiz-Lopez *et al.* 2009). For example, Alcaide et al. (2009)compared wild versus captive and reintroduced Kestrel, but in that instance, it is not clear how many generations the kestrels had bred in captivity. In another case study using experimental captive breeding, Willoughby et al. (Willoughby *et al.* 2017) report on white-footed mice populations that were bred in captivity for 20 generations, and were able to observe mitigating effects on genetic diversity using a very deliberate mating protocol (mean-kinship method). However, in many instances, there may be no choice available as to the source of individuals obtained and used in captive breeding programs. Furthermore, in extreme cases, small numbers of breeding individuals may have to be maintained in captivity for decades until suitable habitat and molecular/technological tools to enable ex situ applications become applicable. From that perspective, it is essential to have realistic estimates of how much genetic

diversity is maintained, and whether estimates of genetic diversity at neutral markers are informative about variation at loci crucial for survival in the wild.

In this study, we used a captive population of the Red Junglefowl (*Gallus gallus murghi*) to characterize the impacts of un-managed captive breeding over a period of 57 years (38-57 generations). The study population called the Richardson Strain (RRJF) has a well-documented history since its import to the United States in 1961. We investigated the consequences of captive breeding on genetic diversity at neutral and adaptive genetic markers experienced by this population over the last 50 years.

Demographic history of Richardson's Red Junglefowl (RRJF)

The study population of Red Junglefowl was initially imported from Northern India in 1961 as part of the U.S. Fish and Wildlife's Foreign Game Introduction Program (FGIP) and documented in USFWS Special Reports (Bump 1964). More recently, the history of the population in the United States was documented in detail in the graduate thesis of Tomas Condon (Condon 2012). Briefly, the birds that were imported to the United States were captured in the Indian states of Bihar and Uttar Pradesh (now Uttarakhand), between 1959-1961. A total of 119 survived the capture and shipping to the United States. These birds were placed in hatcheries across nine different states, and over the next decade, approximately 10,000 birds were released into U.S. habitats for hunting, until the FGIP program ended. Over the next four decades, almost all the descendant RJF a) failed to survive in their new habitats, b) became incorporated into private aviaries, or c) remained in small breeding populations maintained by dedicated researchers and

aviculturists. All the surviving RJF of known ancestry (estimated around 100-150) were derived from a small number of birds. These birds have a documented chain of custody. In 1969, I. Lehr Brisbin (of the Savannah River Ecology Lab) received five birds from the South Carolina Department of Wildlife Resources, to which 26 were added in 1970. By 1971, this flock numbered only eight adult birds (four of each sex). While more hatchlings were added to this in mid-1971, the population remained at under 15 individuals, until they were transferred to Mr. Isaac Richardson of Alabama. Mr. Richardson started his colony with 12 individuals (8 males). Between 1972-2010, the colony was sustained by Mr. Richardson, hence giving this population the name Richardson's Red Junglefowl (RRJF). Over this time, the population expanded and was distributed to a few others, but at any time, the breeding colony never exceeded 20 individuals (Condon 2012). Based on behavioral and morphological studies of Red Junglefowl, it has been argued that the RRJF represent a pure and unique population of Red Junglefowl (Brisbin & Peterson 2007), whose preservation is critical in the face of hybridization with domestic chickens in the native range of Junglefowl (Brisbin 1995; Brisbin & Peterson 2007).

The research colony maintained at Texas A&M University (TAMU) is one of the three known remaining populations that trace their ancestry back to the survivors of Mr. Richardson's flock. The TAMU colony was established with two males and three females and expanded to 27 individuals at the time of the study. All available individuals were included in this present study.

Genetic diversity and effective population sizes

As the management of genetic diversity is one of the primary intentions of captive breeding programs, it is important to assess the consequences of captive breeding over multiple

generations on genetic diversity. Other authors have shown reductions in genetic diversity in captive populations, and these typically result from differential survival and increased genetic drift, but also a form of (unintentional) domestication selection (Briscoe *et al.* 1992; Montgomery *et al.* 2010). In these instances, it is important to characterize what the effective population size is, as this one parameter can drive various other genetic parameter estimates. The effective population size (N_e) can be defined as the size of an ideal population that experiences the same amount of genetic drift or inbreeding as the actual population (Wright 1931, 1948). While a number of approaches have been developed to estimate N_e - for example, temporal estimates based on two or more samples, methods based on linkage disequilibrium, and coalescent approaches (Wang *et al.* 2016), due to our access to a single point in time, as well as our interest in understanding recent effective population size, we focused only on methods for contemporary or recent Ne estimates. In this study, we evaluated genetic diversity, estimated the effective population size (Ne), and analyzed linkage disequilibrium using two types of markers - namely microsatellite markers and SNP markers.

Materials and methods

Study Population

A total of 27 birds bred in captivity at the Texas A&M Poultry Research Center were genotyped in this study. The population represents three generations. The parental cohort was comprised of 2 males and three females in January 2016 and had expanded to 27 individuals by the second half of 2017. At the time of sampling, the population included original parents (used to establish colony), in addition to two subsequent generations that were hatched in Texas. All individuals

available were included in this study.

Molecular Markers

Venal blood was sampled from all individuals by puncturing the brachial vein and stored in Longmire Buffer until further processing. Genomic DNA was isolated from whole blood using the DNeasy Blood and Tissue kit (Qiagen Inc). For microsatellite genotyping, a total of 18 microsatellite markers were randomly selected (from Tadano *et al.*(2007)) and screened for the presence of polymorphic loci. Of the screened loci, three loci were monomorphic, or were not amplified by PCR consistently and were not used further (full list and primer sequences presented in S1). To generate genotype data from microsatellite loci, we used the fluorescently labeled M13 method (Schuelke 2000), which allows greater flexibility of fluorescent dye tagging for multiplexing. PCR thermoprofile was optimized based on published T_m for each primer pair (and considering M13 sequence), and amplification was performed in a 25µl reaction using NEB Taq Polymerase with reaction buffer (New England Biolabs) and NEB dNTPs (New England Biolabs).

Multiplexed samples were prepared for fragment analysis length analysis on the ABI 3730 capillary analyzer, and sent to the DNA sequencing facility on Science Hill (Yale University, New Haven, CT) for fragment analysis. For SNP analyses, equimolar DNA isolates were submitted to the molecular genetics lab at Hy-Line International for genotyping using a custom SNP panel (Fulton *et al.* 2016; Nguyen-Phuc *et al.* 2016). The SNP panel was developed as an assay for the Major Histocompatibility Complex - B locus (MHC-B), based on the KASP^{TM} chemistry (LGC group, UK) where each allele is identified by its fluorescent label (VIC or FAM). A total of 90 known SNP loci are genotyped using this panel and covers the region between basepairs 30,189 to 240,933 on chromosome 16. While microsatellite loci came

from multiple chromosomes, all the SNP loci are from a single gene-rich microchromosome, and as is typical for SNP datasets, the markers represent a mix of intronic/intergenic (putatively neutral) and coding regions.

Genetic Analyses

The raw data was downloaded into the software Geneious (Biomatters, New Zealand) and analyzed using the microsatellite plugin. Allele bins, based on peak data from across all samples, were created for each locus. Following this, every individual biological sample was processed through the peak calling step. Once the automatic peak calls were obtained, every allele call was manually verified to check for errors. The final allele calls were exported into the MS Excel-based tool, GENALEX v6.5 (Peakall & Smouse 2012). The data were then exported to the GENEPOP format for estimation of the effective population size. The SNP calls were generated (by Hy-Line International) using the Kraken software (LGC group, UK). IUPAC base calls were first re-coded manually into 2-letter genotypes, and then imported into GENALEX and recoded into a numeric format for further analysis.

Estimation of expected and unbiased expected heterozygosity (H_e and uH_e), and measures of the inbreeding coefficient F_{IS} were both calculated within GENALEX, based on 999 permutations of the data. These parameters were estimated for both the microsatellite and SNP data independently.

Effective Population Size

Next, to estimate the effective population size of this captive-bred population, we used three different estimators that are known to estimate contemporary or recent effective population sizes - namely the heterozygosity excess (HE) method (Zhdanova & Pudovkin 2008), the linkage disequilibrium (LD) method (Waples & Do 2008)and the molecular coancestry (MC)

method (Nomura 2008). All three N_e estimates were generated using the software program NeEstimator, version 2 (Do *et al*. 2014). These estimators are expected to estimate the population size based on shared alleles (molecular coancestry) or the signal of genetic drift - either due to allele frequency differences among parents (Heterozygote excess) or due to the linkage disequilibrium among markers. In this case, we also know which assumptions were violated with some certainty. For example, this captive population has been closed to immigration and not subject to selection (except unintentional domestication selection in captivity), but the assumption of random mating is likely to be violated, as we expect this population to have an increased frequency of mating among relatives over the last five decades. While it is known that most real natural populations may violate one or more assumptions of an ideal population, the departure from random mating is expected to result in underestimates of N_e (Waples *et al.* 2014). Similarly, the molecular coancestry estimate is expected to be biased downward in inbred populations (Nomura 2008). Considering the differential consequences of these assumptions for our captive population, we estimated N_e using the three methods mentioned above. The analyses were carried out separately for the microsatellite data, and for the SNP data. Furthermore, we were particularly interested in determining the N_e as obtained from markers from a single chromosome. By definition, a chromosome is a single linkage group, and linkage of loci over a single chromosome can be expected to be stabler over evolutionary time, compared to markers sampled from across the genome. However, estimates of contemporary N_e are expected to be biased downward by the length of the chromosome, and also the number of chromosomes (Waples *et al.* 2016) primarily due to physically linked loci, and recombination frequencies being lower on short chromosomes. To our knowledge, this is the first study where a high density of markers from a single chromosome was used to explore the population history.

Haplotype and Linkage Analyses

To understand the effects of captive breeding on the number and structure of unique haplotypes in the captive population, we first phased the SNP genotype data to identify the unique haplotypes present in the population. We used the program PHASE v2 (Stephens *et al.* 2001) to phase the SNP loci into unique haplotype sequences. We performed phasing and estimation of haplotype frequencies from five replicate runs of the program PHASE. Each replicate run started with a different seed and was comprised of 1000 iterations, with a thinning interval of five and a burn-in interval of 100. Following the completion of these runs, the number of unique haplotypes and their frequencies were recorded and checked across replicate runs. As the number of haplotypes and their frequencies was very consistent across runs, we determined that increasing the run length was not necessary (Stephens *et al.* 2001).The list of unique haplotypes was then used to construct a haplotype network using the 'APE' package on the R statistical platform (Paradis *et al.* 2017). This approach constructs a matrix of Kimura-2 parameter genetic distance (Kimura 1980) based on nucleotide differences among the haplotypes and plots a network representing differences among haplotypes.

Finally, to examine linkage structure across chromosome 16 based on the 90 SNP loci, we used the program HaploView (Barrett *et al.* 2005).This program summarizes estimated Minor Allele Frequency (MAF) for each locus and generates haplotype and linkage information for locus pairs, which are then represented in a graphical format. Higher values of linkage disequilibrium (LD) suggest stronger association among loci, and in turn, showing low recombination between loci.

Results

Genetic Diversity

For the microsatellite data, 93.3% of the loci were polymorphic, and the three estimates of heterozygosity (observed, expected, and unbiased) based on microsatellite markers were very similar to each other, ranging from 0.359 to 0.371 to 0.379, respectively. For the SNP dataset, only 37.8% of the loci were polymorphic, which translated into heterozygosity estimates of 0.107, 0.096, and 0.98 (observed, estimated, and unbiased, respectively, Table 1). Estimates of F, the fixation index/inbreeding coefficient, were also divergent between the microsatellite (0.065) and SNP datasets (-0.137). Positive values of F are typically indicative of more significant inbreeding than expected, whereas negative values suggest more outbreeding than expected. This latter result is potentially a consequence of the low observed and expected heterozygosities for the SNP dataset, arising from the small proportion of SNP loci that were polymorphic.

Effective Population Size estimates

The N_e estimates were in the low single digits for the microsatellite marker dataset, except for the heterozygote excess method. For the single chromosome SNP dataset, both the LD and MC estimates were 1 or lower with narrow 95% intervals (Table 2), whereas the H_E estimator was in the low single digits (N_{eHE}=4.8, 95% CI=3.1-11). If these estimates are compared against the total local population (Texas A&M), the N_e/N_c ratios range from about 0.14-0.27 for microsatellite markers, whereas they range from 0.007-0.17 for the SNP based estimates.

Differences between neutral microsatellite markers and SNP markers

Given the known history of the study population, the low N_e estimates were not unexpected, but the differences between the microsatellite markers from multiple locations in the genome, and

the single chromosome SNP markers were notable, and reveal different population histories. Both the MC and the LD methods estimated N_e to be equal to or lower than one, based on the SNP dataset. Whether these differences are the result of different recombination frequencies for marker types or actual biological lineages for unique haplotypes is challenging to differentiate. However, identification of unique haplotypes (next section) partly answers this question.

Unique Haplotypes and Linkage

Analysis of linkage disequilibrium based on the SNP loci on chromosome 16 revealed very high D' values across all pairwise comparisons. Linkage disequilibrium (D') was estimated to have a value of 1, indicating little or no recombination between marker pairs (Figure 1). Such high values indicate that as a consequence of captive breeding, individuals in this population have inherited virtually identical chromosomes (chromosome 16 at least), a phenomenon that would be indistinguishable from the absence of recombination.

Analysis of haplotypes in the study population using the program PHASE revealed five unique haplotypes (Figure 2), and of these only three were found at frequencies higher than 10%, with the most common haplotype represented in 70% of individuals (haplotype 1). The next two most common haplotypes were found at approximately 14% frequency each. These haplotypes suggest at best three unique haplotype lineages contributing to the population and presumably do not reflect novel haplotypes emerged by mutations since the initial population contraction.

Discussion

Genetic Diversity

We found relatively low genetic diversity based on heterozygosity, among both marker types, but estimates of heterozygosity were higher using microsatellite markers compared to SNP loci. This is not a surprising result, as microsatellites may not be highly correlated with underlying genomic diversity (Vali *et al.* 2008), for example, due to ascertainment bias for polymorphic loci. Tokarska et al. (2009) have also shown that in species or populations with low genetic diversity, heterozygous SNP loci may be more informative about population history and structure. In our case, the SNP marker panel included loci from both coding and noncoding regions, but all these markers came from a single chromosome. While it is difficult in our study to determine how the SNP loci may be representative of whole genome diversity, there is no particular reason to believe that chromosome 16 would be experiencing selection or drift in ways that are inconsistent with the rest of the genome. Therefore, we believe that the measures of genomic diversity based on the SNP panel to be more informative about genome-wide patterns of diversity, while the microsatellite-based estimates are consistent with and relevant to our understanding of neutral diversity.

The second takeaway of such a comparison between microsatellite markers and SNP markers is the potential implications of such disparity for other endangered or threatened species of conservation interest. Several studies have used microsatellite markers to assess genetic variation following population contractions (Athrey *et al.* 2011; Bouzat *et al.* 1998; Hammerly *et al.* 2013; Hoelzel *et al.* 2002; Johnson & Dunn 2006; Miao *et al.* 2016; Wisely *et al.* 2002). If microsatellite-based measures of genetic diversity are higher than SNP-based whole-genome genetic diversity**,** as observed in the current study, it would be a potentially worrying trend from

the perspective of measuring the genetic viability of wild populations. Due to the relatively low cost and accessibility of microsatellite loci, the usage of these markers has become ubiquitous in population and conservation genetics, and in the assessment of genetic diversity. If high microsatellite variability does not correlate positively with genome-wide diversity, then management decisions need to consider the potential limitations of such data.

Effective Size estimates

We found low (single digit) N_e estimates across all estimators. On the one hand this is consistent with the known demographic histories, but on the other hand, this may also be explained by biases arising from sample size or other assumptions. We expect the N_e estimates to be biased downward due to the small sample size in this study, but these values are not unusual in endangered populations. Several of the N_e estimators expect biased estimates at sample sizes under N=50. However, the sample size in this study included 100% of available individuals, which in this case was the total census population. Another source of bias, especially for the LD method, as reported by Waples *et al.* (2016), is the number of length of chromosomes. In our case, the SNP data were generated from chromosome 16 - one of the smaller micro-chromosomes, which has an estimated length of 48-80cM (Burt & Cheng 1998; Groenen *et al.* 2000). We expect both the smaller sample size and the use of single chromosome SNP markers to be contributing to low N_e estimates using the LD, and perhaps the MC method.

Due to our exact knowledge of the census size, the N_e/N_c ratios are informative about both the processes of marker inheritance, as well as the performance of the estimators themselves. Secondly, all the three estimators used in this study are expected to represent the previous or the

most recent parental generations. Therefore, for both the LD and MC methods, we believe that the estimates obtained here represent the founding event of the Texas population in 2016. On the other hand, as changes in heterozygosity occur more gradually over time, and the accumulation of inbreeding may take several generations, the HE estimator may be more representative of a founder event not in the immediate past, but bounded by the initial founding of the U.S. populations in 1961.

Haplotype diversity and linkage

We observed only three major haplotypes in the population, and it will be valuable to understand how these haplotypes are maintained in subsequent generations. We believe that the low haplotype diversity is a consequence of the history of captive breeding among a small number of individuals, rather than a reflection of the source populations. Haplotype diversity in other wild junglefowl populations has been reported to be quite high. For example, Nguyen-Phuc et al. (2016)reported 313 unique haplotypes from 199 wild-caught individuals from Vietnam, which indicates that the progenitors of the RRJF came from a similarly diverse population, which have since lost genetic diversity in captivity. It is also worth noting that the genetic bottleneck and the resulting reduction in genetic diversity in only 50 years perhaps mirror the early genetic history during selection for domestication, except for periodic gene flow from wild individuals. It would appear that such immigration was necessary to maintain the high levels of genetic diversity that is found in domestic chickens today (Blackburn 2012; Fulton *et al.* 2016; Granevitze *et al.* 2007).

We observed high linkage disequilibrium among loci in the SNP dataset - an estimate that is known to be inflated when using small sample sizes, and very likely to be contributing to the

high D' values observed (Ardlie *et al.* 2002; England *et al.* 2006). Taken together with the reduced haplotype diversity in this population, however, the linkage disequilibrium estimates are biologically plausible.

Selection for tameness and success in captive environments is inevitable in captive breeding populations (Frankham *et al.* 2002; Frankham *et al.* 1986). The genetic consequences of domestication have received much attention in recent years (Wright *et al.* 2010) and the genetic architecture of traits - mainly driven by linkage and pleiotropy are considered to be crucial in the expression of specific phenotypes in domesticated varieties. For example, Wright et al. (2010)showed the presence of strong linkage blocks, as well as low heterozygosity regions associated with selective sweeps in domesticated varieties of chicken. The haplotype diversity and linkage patterns we observed in our captive population appear to be consistent with what might occur early during domestication.

Conclusions

Our study showed the dynamics of genetic diversity and effective population sizes in a small captive breeding bird population. Furthermore, our study was able to relate effective population sizes to the known demographic history of the captive population. We found that the demographic history corresponds to a great extent with the genetic estimates. Furthermore, the low genetic diversity, and haplotype diversity both indicate that these populations are indeed pure, and perhaps unmixed with domestic varieties, since their initial importation to the United States. Our study shows the rapid loss of genetic diversity in captive populations in only a few generations (37-50), which can be particularly concerning from the standpoint of *ex situ*

conservation goals. Finally, we showed how captive breeding populations, even in the absence of any intentional selection, can affect linkage structure across a set of adaptive genetic loci, and potentially reduce the fitness and adaptability of captive-born individuals upon release back into the wild.

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Tables

Table 1: Estimates of genetic diversity for both microsatellite and SNP datasets are presented. For each marker, genetic diversity measures are shown along with their standard errors. The columns present the number of alleles (Na), effective number of alleles (Nae), observed heterozygosity (Ho), expected heterozygosity (He), the unbiased expected heterozygosity (uHe), and the fixation index F.

Marker Type		Na	Ne	Ho	He	uHe	F
Microsatellite	Mean	2.33	1.74	0.36	0.37	0.38	0.07
	SE	0.16	0.14	0.06	0.05	0.05	0.08
SNP	Mean	1.38	1.13	0.11	0.1	0.1	-0.14
	SE	0.05	0.02	0.01	0.01	0.01	0.02

Table 2: Estimates of effective population size Ne are presented for microsatellite and SNP datasets. Ne estimates from three estimators are shown along with their respective 95% confidence intervals. Ne was estimated using the Heterozygote Excess (NeHE), Linkage Disequilibrium (NeLD), and the Molecular Coancstry (NeMC) methods.

	NeHE	95% CI	NeLD	95% CI	NeMC	95% CI
Microsatellite	inf	7.3-inf	7.3	3.6-12.5	4	1.1-8.8
SNP	4.8	3.1-11	0.2	0.1-0.2	1	1-1

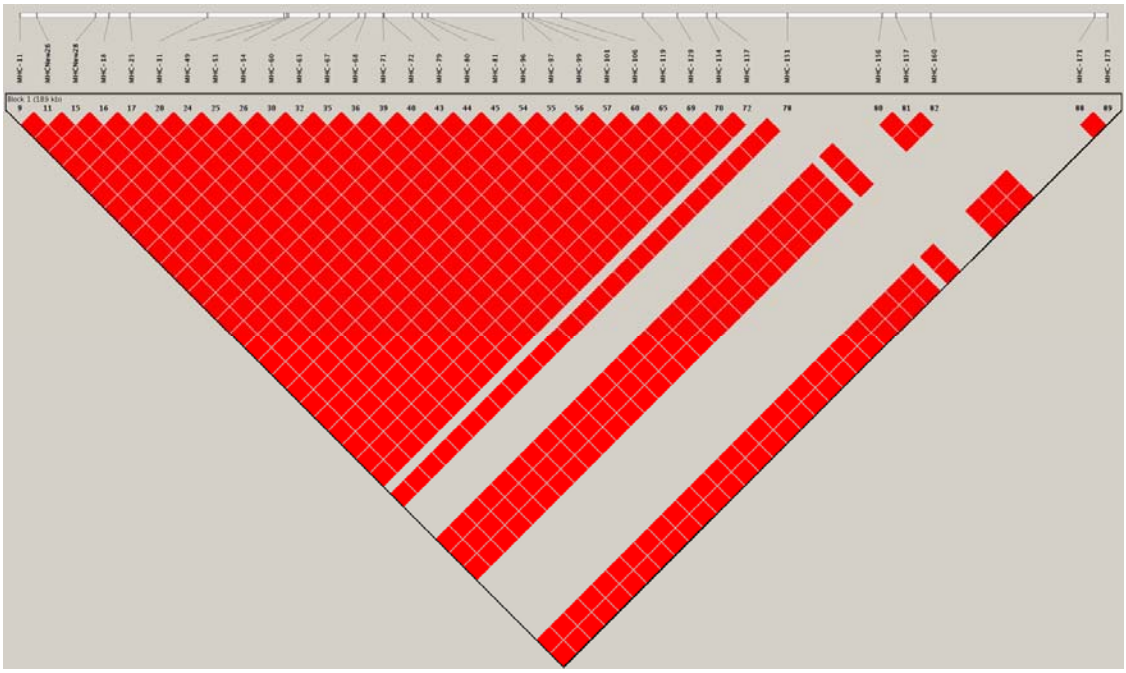

Figure 1: A linkage map based on the 90 SNP loci on chromosome 16, generated from genotype data for the study population. The map shows pairwise estimates of linkage disequilibrium between markers. Red colored blocks suggest high linkage disequilibrium (D') values of 1, implying little to no recombination between markers.

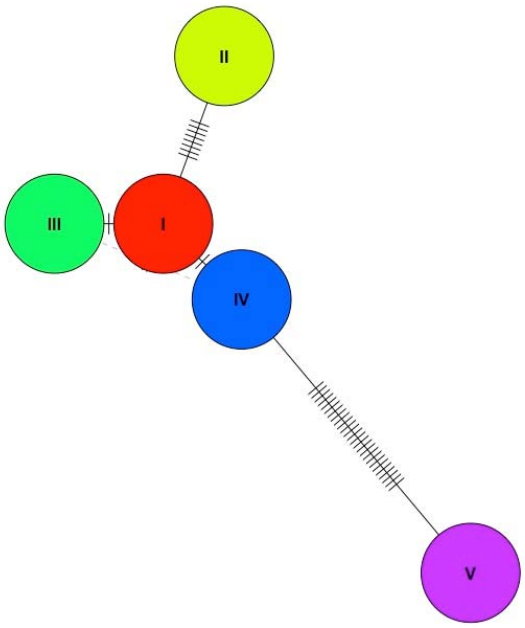

Figure 2: A haplotype network of the the five (phased) haplotypes detected in the study population. Only three of the five unique haplotypes were found at a frequently of over 10% in the population (I, III, and V).